Genetic approaches to somatosensation

Bjarke Abrahamsen

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Department of Biology
University College London
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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Three approaches to understanding peripheral pain pathways are described in this thesis. Cre-loxP technology has been used to delete genes specifically in specialised sensory neurons that respond to tissue damage (nociceptors). The sodium channel selective $Na_v1.8$ promoter is used to drive Cre expression. I used a mouse expressing diphtheria toxin A-chain downstream of a floxed stop signal, crossed with the $Na_v1.8$Cre mouse to delete all cells expressing this channel. This lead to massive cell loss of nociceptive neurons, associated with altered pain behaviour. The mice lost cold, mechanical and inflammatory pain, but not thermal or neuropathic pain behaviour. These observations are consistent with modality specific pain pathways in the peripheral nervous system. Microarray analysis was used to identify the transcripts selectively expressed in the lost sensory neurons, providing potential new analgesic drug targets for inflammatory pain.

In a further study exploiting knock out mice, I identified a line that had lost sensitivity to light touch, but was otherwise normal in terms of pain pathways. This sensory loss was due to the insertion of a LacZ cassette in a gene encoding Papin, a PDZ-protein of unknown function. Further analysis showed a loss of interactions between sensory neurons and mechanosensitive Merkel cells, and a loss of sensory neuron numbers.

Finally, conditional deletion of $Na_v1.7$ in mice, and global loss of $Na_v1.7$ in man have been shown to lead to a loss of pain sensitivity. I used a recently discovered peptide blocker of $Na_v1.7$ to examine the role of this channel in normal mice. I found that $Na_v1.7$ block leads to a loss of mechanical, inflammatory and neuropathic pain, making this sodium channel a very attractive analgesic drug target.

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This thesis is dedicated to Bent.

1 INTRODUCTION

1.1 PAIN

Pain, defined as an emotional and sensory experience caused by potential or actual tissue damage (IASP, 1994), is one of the most common clinical conditions. According to a survey from the British Pain Society, almost 10 million people in the UK suffer some sort of pain every day (BPS, 2005). Physiological pain refers to pain evoked by a painful stimulus e.g. a needle prick. The information from the insult will go to the brain and act as a warning to prevent tissue damage. Pathophysiological pain is due to a condition such as inflammation, nerve damage or nerve dysfunction. The current pharmacological pain-treatment is non-opioid based (paracetamol, cyclo-oxygenase inhibitors etc.); opioid based (codeine, morphine, deamorphine etc.) as well as agents whose primary indication is for conditions other than pain, but have proven useful in the treatment of pain conditions (anticonvulsants, antidepressants, steroids and others).

An improvement in the treatment of pain is needed, as the daily experience of pain can have a devastating effect upon individuals' quality of life and the current options for treatment often fail. A way to achieve this is to investigate the molecular mechanisms of pain to obtain a better understanding and through that find ways to develop better treatments.

1.2 PRIMARY SENSORY NEURONS AND NOCICEPTORS

The primary afferent sensory neurons, Dorsal Root Ganglion (DRG) neurons and trigeminal neurons terminate in the periphery and work as sensors, transducing noxious and non-noxious stimuli into action potentials. The signal is carried by the axon to the central synapse where secondary and tertiary neurons project the signal for further processing in the brain where pain is perceived.

The primary sensory neurons are myelinated to different degrees: the most heavily myelinated are the Aβ-fibres, followed by the Aδ-fibres that are only lightly myelinated and the C-fibres are unmyelinated. These neurons can also be characterised by their conduction

velocity: Aβ>10 $msec^{-1}$, Aδ at 1.2-10 $msec^{-1}$, and C-fibres at <1-2 $msec^{-1}$. The Aβ-fibres respond very fast and mainly to innocuous stimuli. It has been reported, however, that a small population of the Aβ-fibres might be involved in nociception (Djouhri and Lawson, 2004;Lawson, 2002). In contrast, the Aδ-fibres and C-fibres exhibit a much slower conduction of information. The C-fibres respond to noxious insults, transducing thermal, mechanical and chemical stimuli into trains of action potentials and are called nociceptors. Under normal conditions nociceptors may be inactive but if the tissue is exposed to stimulation strong enough to introduce damage nociceptors will be activated. A large group of specialised receptors and proteins transduce and regulate the sensitivity of nociceptors – see fig.1.

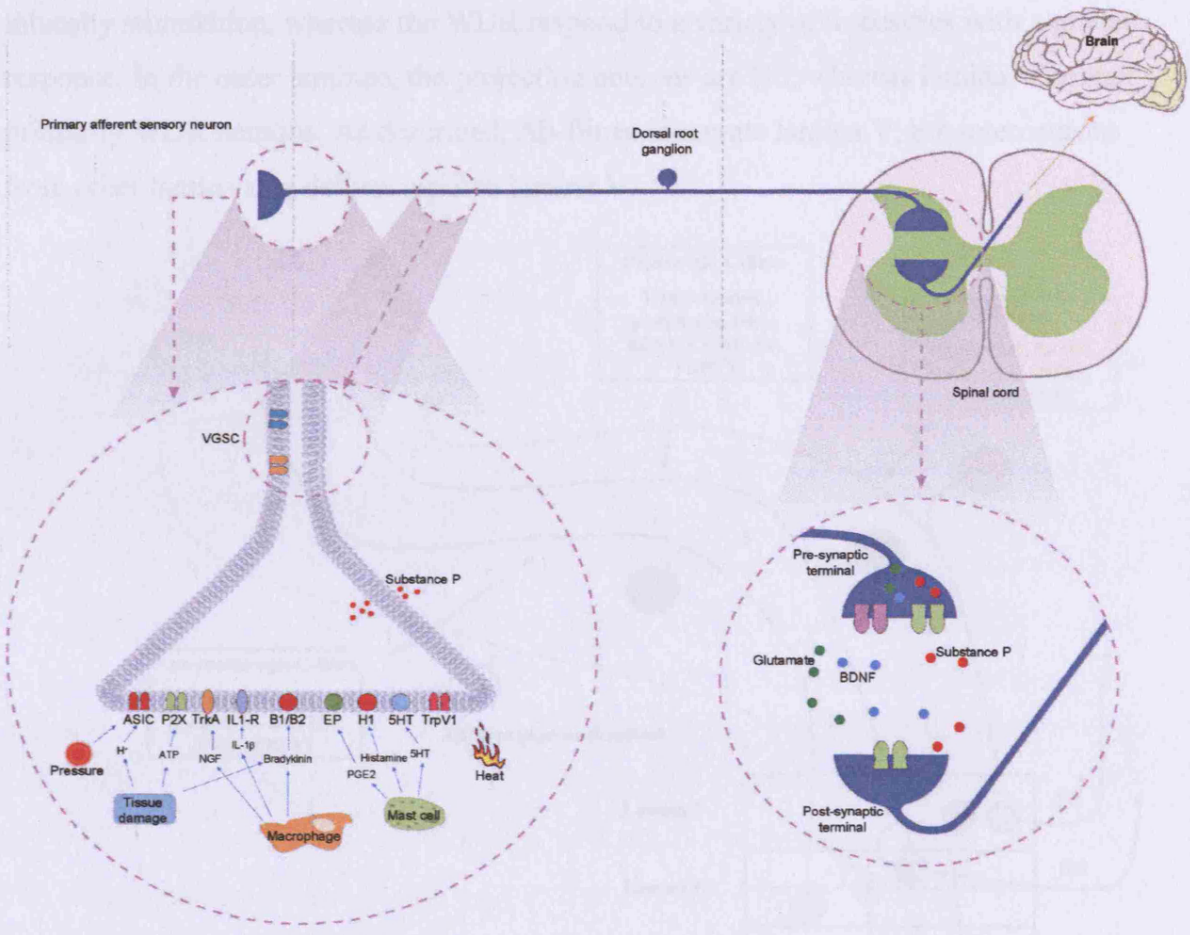

Figure 1. The primary sensory neurons.
Information from the nerve terminals are transduced into electrical signals and carried by the dorsal root ganglia to dorsal horn of the spinal cord where the neurons synapse onto the secondary neurons from where the signal is carried to the sensory cortex. At the nerve terminal a number of receptors are located sensing tissue damage and controlling the flux of ions over the membrane generating action potentials. Adapted from Anemone Labs (2007)

At the dorsal horn of the spinal cord, termination of 1st order neurons occurs at different depths. The dorsal horn is divided into laminae I-X (see fig.2), with the five most superficial layers involved in transmission of sensory information. The Aβ-fibres terminate in the deep laminae III and IV, whereas the Aδ-fibres terminate in laminae I and V. Finally, the C-fibres terminate in the superficial lamina I and II (Snider and McMahon, 1998). The sensory neurons can synapse with two different sets of projection neurons, nociceptor-specific (NS) or wide dynamic range (WDR) neurons. The NS respond only to high-

intensity stimulation, whereas the WDR respond to a variety of intensities with a graded response. In the outer laminae, the projecting neurons are NS, whereas lamina V contains primarily WDR neurons. As described, Aβ-fibres innervate lamina V, but interneurons from other lamina also deliver input to lamina V.

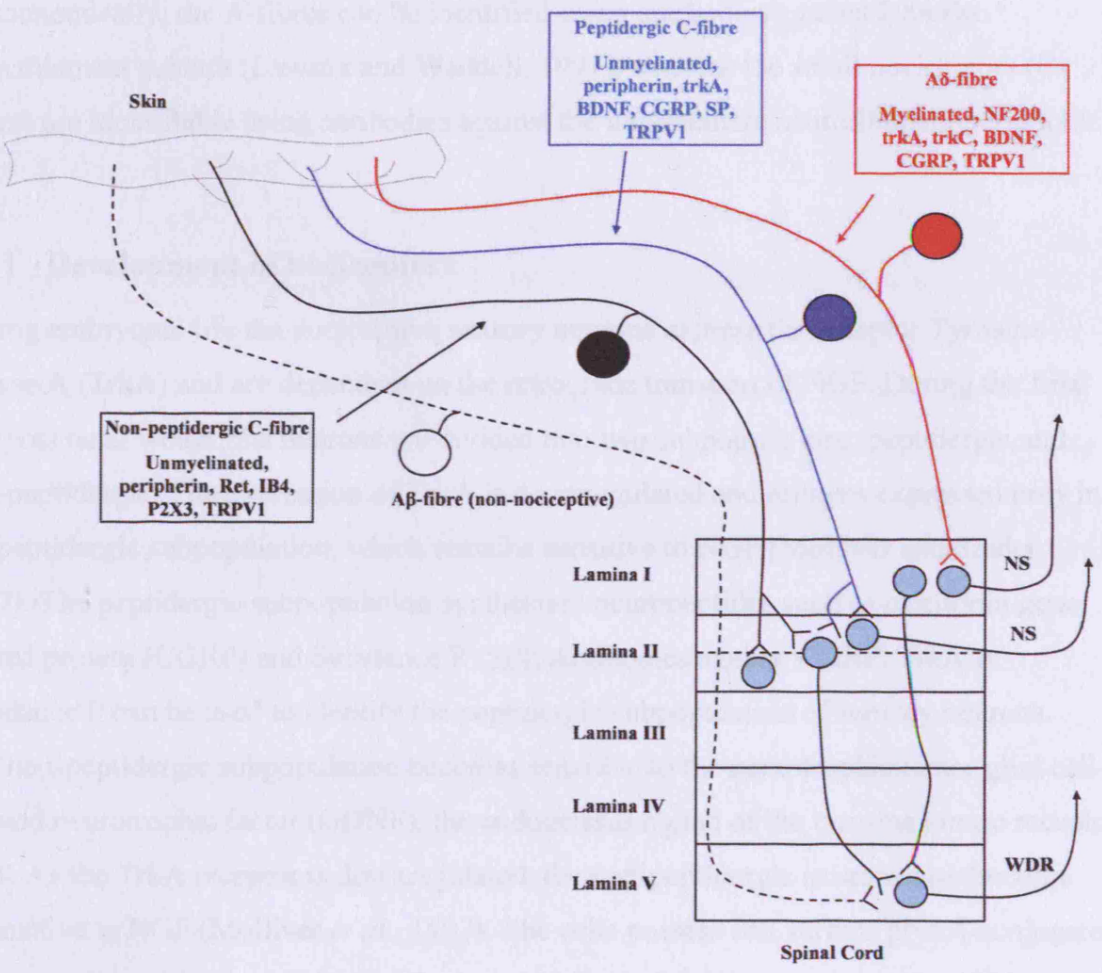

Figure 2. The termination of sensory neurons on the dorsal horn. Adapted from Foulkes and Wood, 2008.

1.3 NOCICEPTION

The Aδ-fibres and C-fibres, the two subpopulations of primary afferent neurons that primarily are involved in nociception, are polymodal. When noxious insults are introduced, specialised receptors expressed in the nerve terminal become activated and allow the movement of ions over the membrane, which produces a depolarisation resulting in the generation of action potentials.

The endings of the nerves differ between the different subpopulations. The Aβ-fibres ends in specialised organs whereas the majority of the Aδ and the C-fibres terminate as free nerve endings in the skin (MacIver and Tanelian, 1993).

Histochemically, the A-fibres can be identified using antibodies against 200kDa neurofilament subunit (Lawson and Waddell, 1991), whereas the small nociceptors (C-fibres) are identifiable using antibodies against the intermediate neurofilament peripherin.

1.3.1 Development of nociceptors

During embryonic life the nociceptive sensory neurons express the receptor Tyrosine Kinase A (TrkA) and are dependent on the retrograde transport of NGF. During the first two post natal weeks, the neurons are divided into two subpopulations, peptidergic and non-peptidergic. The expression of TrkA is downregulated and remains expressed only in the peptidergic subpopulation, which remains sensitive to NGF (Molliver and Snider, 1997). The peptidergic subpopulation synthesises neuropeptides such as calcitonin gene-related protein (CGRP) and Substance P (SP). Antibodies against CGRP, TrkA or Substance P can be used to identify the peptidergic subpopulation of sensory neurons. The non-peptidergic subpopulation becomes sensitive to the neurotrophic factor glial cell-derived neurotrophic factor (GDNF), the endogenous ligand of the tyrosine kinase receptor, RET. As the TrkA receptor is downregulated, the non-peptidergic neurons also become insensitive to NGF (Molliver *et al.*, 1997). The cells possess cell surface glycol-conjugates that can be identified by binding of the lectin Isolectin B4 from *Griffonia simplicifolia* (Silverman and Kruger, 1990).

1.3.2 Mechanical nociception

Mechanosensation is part of human daily life giving the ability to sense touch, texture, sound etc. Mechanosensation is regulated through two different sets of mechanoreceptors, low threshold mechanoreceptors (LTM) that respond to innocuous stimuli and high threshold nociceptors that respond to noxious mechanical stimuli. The following part will focus on high threshold mechanosensation (HTM).

A conopeptide, NMB-1, was shown by Drew et al. (2007) to block pressure evoked pain in rats. When biotinylated NMB-1 was co-stained with Peripherin nearly all cells that had bound MNB-1 also expressed peripherin, linking the expression to the nociceptive population of sensory neurons (Drew *et al.*, 2007). Many hypotheses of possible transducers of intense mechanical stimulation have been tested, but so far very few proteins have proven to be strongly involved in mechanical nociception. Among the candidates are certain cation-permeable ion channels belonging to the large family of Transient Receptor Potential channels (Trp).

One of the Trp-channels suggested to be involved is $TrpV_4$. It is expressed mainly in the small C-fibres (Nagata *et al.*, 2005) and in free-nerve endings (Suzuki et al., 2003b). A $TrpV_4$ knockout mouse did show impaired response to noxious tail pressure (Suzuki et al., 2003a). Another Trp-channel possibly involved in mechanical nociception is $TrpA_1$. Two different $TrpA_1$ knockout mice have been generated, but only one mouse was tested for noxious mechanical responses. Unfortunately the testing of the $TrpA_1$ KO mouse was done in a slightly unconventional way, so it makes it difficult to compare the data (Kwan *et al.*, 2006). The study used von Frey filaments, finely graded filaments applied to the paw, and the Randall Selitto apparatus, a blunt incremental pressure applied to paw or tail, to test the mechanical responses. In particular for the von Frey tests, a range of filaments was applied to the paw a total of eight times (1/second) and if the mouse responded more than twice it was considered as a positive response. The Randall Selitto apparatus was used to apply noxious pressure to the paw of the mouse. The applications of von Frey filaments differed from most other studies and the application of Randall Selitto apparatus to the paw of the mouse is a very difficult task. Results appeared to show a resistance to noxious mechanical stimulation (Kwan *et al.*, 2006).

1.3.3 Thermal nociception

For the transduction of thermal stimuli non-specific cation channels belonging to the super family of Trp-channels have so far been the primary candidates. $TrpV_1$-V_4, together with $TrpM_8$ and $TrpA_1$, have been named the "ThermoTrps". In general the $TrpV_{1-4}$ - channels share a high sequence homology of about 40-50 percent. They exist as homomultimers

although the existence of heteromultimers of the $TrpV_1$-$TrpV_3$ has been reported (Hellwig *et al.*, 2005).

$TrpV_1$ has long been thought to be the transducer of noxious thermal heat. The channel is located in the small and medium diameter sensory neurons and recent studies have shown low levels of expression in the brain and in non-neuronal tissue (Caterina, 2007). Initially, the ion channel was cloned characterised as the receptor for the vanilloid, Capsaicin (chilli pepper). Using HEK293 cells expressing $TrpV_1$, a large increase in calcium influx after raising the temperature from 22°C to 45°C suggested involvement in heat sensation (Caterina *et al.*, 1997). A few years later functional data from a $TrpV_1$ KO was published giving further evidence of involvement in heat sensation. The KO mouse showed a very significantly impaired response to thermal stimulation both acute and after induction of inflammation (Caterina *et al.*, 2000). Interestingly, no acute pain phenotype was recorded from a second $TrpV_1$ KO mouse, merely confirming the electrophysiological data and thermal phenotype seen after induction of inflammation (Davis *et al.*, 2000).

Using the $TrpV_1$ sequence for screening, another ion channel was identified and cloned: $TrpV_2$ (Originally called VRL_1). The distribution was restricted to the medium and large diameter neurons and electrophysiological data from HEK293 cells and *Xenopus* oocytes showed activation at temperatures higher than 53°C (Caterina *et al.*, 1999). So far, no data from a $TrpV_2$ null mouse has been published, but a full investigation of thermal responses would be very interesting.

More recent discoveries have identified a further two Trp-channels contributing to sensation of heat. $TrpV_3$ showed a different profile to that of $TrpV_1$ and $TrpV_2$ as it is predominantly expressed in the keratinocytes and not in the sensory neurons, suggesting the skin can detect heat and synapse between keratinocytes and the primary neurons (Xu *et al.*, 2002;Peier *et al.*, 2002b). The activation of $TrpV_3$ occurs already at innocuous temperatures around 33°C. The $TrpV_3$ KO mouse showed a strong resistance to innocuous heat and a very strong resistance to noxious heat measured above 50°C (Moqrich *et al.*, 2005). Interestingly, responses to noxious heat after inflammation showed no differences.

Further, $TrpV_4$ was identified as sharing its characteristics with $TrpV_3$, being mainly expressed in keratinocytes and not sensory neurons, and becoming activated at innocuous temperatures. In both HEK293 cell systems and *Xenopus* oocytes, $TrpV_4$ heat-evoked currents are detectable at temperatures above 27°C. (Guler *et al.*, 2002). As with $TrpV_1$, a strong reduction in responses to noxious heat after inflammation was also reported (Alessandri-Haber *et al.*, 2006)

$P2X_3$, belonging to the purinergic receptor family and is activated by ATP and expressed in small sensory neurons (Chen *et al.*, 1995), has been shown to have a possible role in thermal sensation. Performing electrophysiology on the $P2X_3$ knock out mouse showed a significant deficit in thermal sensations but this was not reproduced *in vivo*, testing thermal responses using the hot-plate and tail flick (Souslova *et al.*, 2000). Electrophysiological testing of DRG neurons confirmed that P2X-receptors expressed in DRG are responding to temperature dependent ATP release, but the range of temperatures applied were between 25-40°C and not in the noxious range (Khmyz et al., 2007).

With low temperature stimuli, innocuous and noxious cold sensation, two other Trp-channels have been identified; $TrpM_8$ and $TrpA_1$. Both are expressed in the small neurons in the DRG and are activated at temperatures below 25°C and 18°C respectively (Story *et al.*, 2003). After the cloning of $TrpM_8$ by Peier et al 2002, $TrpM_8$ was expressed in CHO cells and showed, using calcium imaging, that lowering the temperature to 15°C increased the calcium levels in $TrpM_8$- expressing cells (Peier et al., 2002a). Functional data from transgenic $TrpM_8$ knockout mice from two independent groups demonstrated resistance to temperatures below 15°C (Colburn *et al.*, 2007;Dhaka *et al.*, 2007). $TrpA_1$ has, as mentioned above, shown a possible involvement in mechanical nociception. Another interesting role for $TrpA_1$ could be in cold nociception demonstrated by cold activation of $TrpA_1$ when expressed in CHO cells and *Xenopus* oocytes (Story *et al.*, 2003). Two knockout mouse lines were generated and interestingly, conflicting observations in response to cold were recorded (Bautista *et al.*, 2006;Kwan *et al.*, 2006). Kwan et al. found differences between WT and KO in two models of cold stimulation, using cold plate and cooling by evaporating of acetone applied to the paw. In contrast, the KO from Bautista et al. found no differences at all due to cold, but a different regime of studies was applied. Bautista et al. used the latency in cold plate behaviour to determine a difference between

control and KO mice compared with Kwan et al's approach where measuring the number of pain behaviours over a period of 5 minutes was applied. The evaporation study by Kwan et al. involved application of the acetone and counting pain behaviour immediately for two minutes; Bautista et al. measured the cumulative time spent licking and biting.

1.3.4 Inflammatory pain

After a noxious insult strong enough to introduce tissue damage, an inflammatory response will normally occur. Inflammatory modulators are released from the surrounding tissue activating and sensitising the sensory neurons leading to *Allodynia*, a painful feeling from a stimulus that would not normally be painful, or *Hyperalgesia*, where an increased response is seen to a painful stimulus.

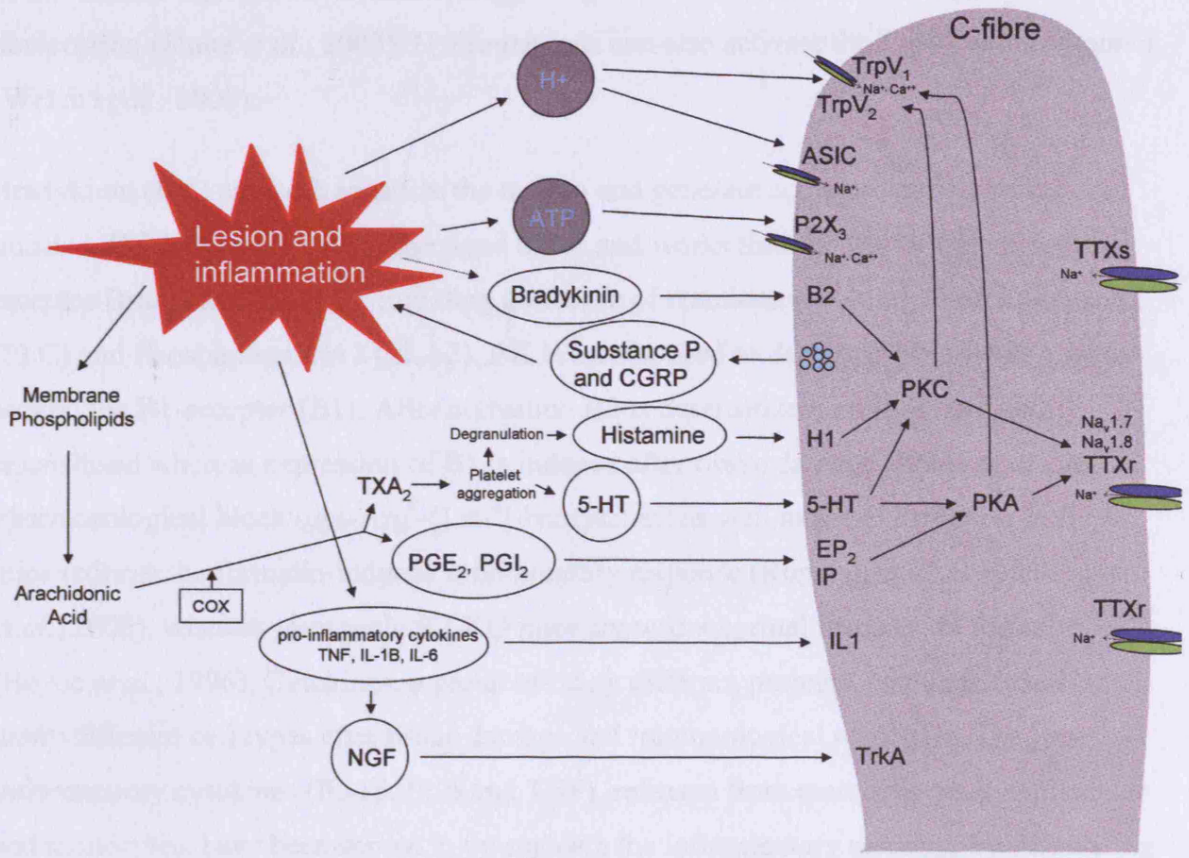

Figure 3. Overview of the inflammatory response.
Due to tissue damage released compounds works through their respective receptors activating or sensitizing the neuron.
TTXs/r: Tetrodotoxin sensitive/resistant
Modified from (Coutaux et al., 2005)

1.3.4.1 Inflammatory modulators

ATP is released from the cytosol of damaged keratinocytes and acts on P2X purinoceptors (Cook and McCleskey, 2002). Similar to ATP, hydrogen ions (H^+), also released upon tissue damage, can also generate action potentials if injected on their own. A lowering of the pH activates Acid Sensing Ion Channels (ASIC) of which isoforms 1-3 are expressed in the sensory neurons. Knockouts of ASIC1 and ASIC2 showed no deficit in nociception, whereas deletion of the ASIC3 demonstrated a clear pain resistant phenotype to intramuscular injected acidic saline, suggesting an involvement in inflammatory nociception (Sluka *et al.*, 2003). Hydrogen ions can also activate the $TrpV_1$ cation channel (Welch *et al.*, 2000).

Bradykinin (BK) can both sensitise the neuron and generate action potentials when injected. BK is released by the damaged tissue and works through the G-protein coupled receptor Bradykinin B2 (B2), initiating a cascade of reactions involving Phospholipase C (PLC) and Phospholipase A2 (PLA2). BK is metabolised to des-[Arg^9]-bradykinin which acts on the B1-receptor (B1). After activation B2 is desensitized, endocytosed and resensitised whereas expression of B1 is induced after tissue damage (Prado *et al.*, 2002). Pharmacological block (des-Arg^9-[Leu^8]-bradykinin) as well as gene disruption of B1 in mice reduces the formalin-induced inflammatory response (Rupniak *et al.*, 1997;Pesquero *et al.*, 2000), whereas transgenic B2-KO mice showed a normal response to formalin (Boyce *et al.*, 1996). Cytokines, a group of many different proteins, can be released by many different cell types after tissue damage and immunological responses. The pro-inflammatory cytokines (IL-1β, IL-6 and TNF), released from macrophages, lymphocytes and monocytes, have been shown to up-regulate the inflammatory response by stimulating the release of prostaglandins (Copray *et al.*, 2001;Cunha *et al.*, 2005b). Prostaglandins are known to sensitize sensory neurons via specific receptors. Due to inflammation, phospholipase A2 hydrolyses membrane phospholipids and arachidonic acid is released. It is further converted by cyclooygenases (COX) into a prostaglandin intermediate, and specific isomerases generate the different prostaglandins. The prostaglandins PGE2 and PGI2 work through the EP1, EP2 and IP receptors (Zeilhofer, 2007) or potentiate other receptors such as $TrpV_1$ (Moriyama *et al.*, 2005). The neurotransmitter Serotonin is found both in the CNS and PNS and in the sensory neurons; studies have shown strong involvement in nociception via the $5HT_3$ receptor. Zeits et al. analysed the 5-HT_3 null

mouse and found that the 5-HT_3 receptor is expressed in both small C-fibres and in some medium and large fibres. Further, in the formalin test they showed that deletion of the 5-HT_3 receptor reduced the response in the second phase (Zeitz *et al.*, 2002). The action of histamine in the CNS is most interesting as all three identified and cloned histamine receptors (H1-3) are distributed throughout the CNS. Blockade and transgenic deletion of the H1 receptor showed reduced sensitivity to both acute nociception and inflammatory nociception (Mobarakeh *et al.*, 2000;Olsen *et al.*, 2002). Studies of the H2 receptor null mouse show some involvement of the receptor in acute nociception, but not in inflammatory nociception (Mobarakeh *et al.*, 2006). H3 is expressed in the sensory neurons, located on the small and medium fibres (Cannon *et al.*, 2007a). Interestingly, pharmacological blockade of H3 has an antinociceptive effect, reducing swelling and nociceptive responses (Cannon *et al.*, 2007b). Neurotrophins work through the Tyrosine Kinase Receptors (Trk) expressed on the terminals of sensory neurons which activate different signalling pathways. NGF works through the TrkA receptor and regulates expression of many proteins such as SP and CGRP, ion channels and receptors. NGF levels are increased under inflammatory conditions and several studies show the involvement of NGF in inflammatory nociception (Reviews; (McMahon, 1996;Pezet and McMahon, 2006). Synthesis of BDNF is also upregulated in the DRG after inflammation, but it is transported to the nerve terminals in the dorsal horn where it works on TrkB (Lee *et al.*, 1999). The neuropeptide Substance P is expressed by C-fibres and released into the dorsal horn not only in inflammation, but in most nociceptive modalities. It acts centrally on the NK-1 receptor and regulates the excitability of the dorsal horn neurons. Studies on the NK-1 knockout mouse showed a strong reduction in responses to inflammation (Kidd *et al.*, 2003). CGRP is expressed abundantly in the sensory neurons, transported to the nerve terminals and released into the dorsal horn synapse (Gibbins *et al.*, 1987;Gibson *et al.*, 1984) and acts through a seven transmembrane GPCR multi subunit complex (McLatchie *et al.*, 1998). After inflammation CGRP levels have been shown to be increased (Hanesch *et al.*, 1993).

1.3.5 Important ion channels in inflammatory pain

1.3.5.1 $Na_v1.7$

A tissue-specific DRG knockout of the $Na_v1.7$ showed a large reduction in the response in the second phase of the formalin test. When exposed to long term inflammation using CFA no change in response to either thermal and mechanical stimulation was recorded suggesting a major role of $Na_v1.7$ in inflammatory pain (Nassar *et al.*, 2004).

1.3.5.2 $Na_v1.8$

Inflammatory modulators can increase the activity of PKA and PKC and it has been shown that both kinases can regulate $Na_v1.8$ (Vijayaragavan et al., 2004), but the responses from the $Na_v1.8$ null mouse tested after induction of inflammation using carrageenan and Formalin, recorded only a delay in developing hyperalgesia was recorded in the KO compared with controls (Akopian *et al.*, 1999;Nassar *et al.*, 2005)

1.3.5.3 $TrpV_1$

One important ion channel in inflammatory pain is the $TrpV_1$. In two transgenic mouse $TrpV_1$ knockout lines, thermal responses after inflammation were significantly reduced (Davis *et al.*, 2000;Caterina *et al.*, 2000). The channel is sensitised (phosphorylated) by PKC and PKA (Bhave et al., 2002;Premkumar and Ahern, 2000), desensitised by cAMP dependent protein kinases (Mohapatra and Nau, 2005) and activated directly by a number of inflammatory mediators such as protons and prostaglandins.

1.3.5.4 $TrpV_2$

As $TrpV_1$ is so strongly activated by inflammation and contributes to increased thermal sensations, $TrpV_2$ could also be involved. Even though little data is available and no knockout mouse has been generated, data from a selective activator of $TrpV_2$, Probenocid, was published recently. Wild type mice treated with Probenecid showed a very strong inflammatory reaction after injections of carrageenan compared with controls injected only with carrageenan (Bang *et al.*, 2007). This suggests that $TrpV_2$ does contribute to inflammatory pain.

1.3.5.5 $TrpA_1$

As mentioned above, $TrpA_1$ is expressed in the small C-fibres. Specific prostaglandins, PGE_2 and PGD_2, have been shown to activate $TrpA_1$ (Taylor-Clark et al., 2007). An even stronger reduction in the formalin test was seen in the $TrpA_1$ KO mouse. In the first phase of the formalin test, the $TrpA_1$ KO mouse showed around 80 percent reduction in response. In the second phase, which is considered an inflammatory response (Jeong and Holden, 2007), the nociceptive behaviour was almost ablated (McNamara *et al.*, 2007).

1.3.5.6 $P2X_3$

In sensory neurons, $P2X_2$ and $P2X_3$ are both expressed (Chen *et al.*, 1995;North, 2004). They can exist as homomeric ion-channels or as a heteromeric $P2X_{(2/3)}$ (Grubb and Evans, 1999). Electrophysiological studies proved a role for P2X receptors in the nociceptive responses to inflammation and also showed that both $P2X_2$ and $P2X_3$ were upregulated after inflammation, potentiated by ATP and Bradykinin (Paukert et al., 2001;Xu and Huang, 2002). The transgenic $P2X_3$ null mutant mouse showed a reduced response in the formalin test together with reduced responses to noxious thermal stimulation (Souslova *et al.*, 2000).

1.3.6 Neuropathic pain

Pain perception initiated or caused by primary lesions or dysfunction in the nervous system is defined as neuropathic pain and results in the development of spontaneous and abnormal activity in injured and uninjured afferent neurons (Ji and Strichartz, 2004).

The characteristics of neuropathic pain are an persistent pain characterised by burning pain, electric shock-like pain and pain paroxysms including allodynia, hyperalgesia and secondary hyperalgesia, hyperalgesia in non-injured tissue surrounding the injury (Jensen *et al.*, 2001;Nozaki-Taguchi and Yaksh, 1998). This is caused by host of events including inflammatory mediators released shortly after the neuronal damage, and the central changes induced caused by changes in the retrograde supply of neurotrophic factors GDNF and NGF.

Early work by Bennett et al. tested the effects of intrathecal NGF and GDNF on nerve cells at DRG and spinal level after axotomy (excision of the nerve). GDNF was shown to suppress the reduction in IB4-positive profiles, whereas NGF, to a lesser extent, was shown to suppress a reduction in TrkA positive cells. This suggest that the loss of GDNF and NGF supply after nerve injury is responsible for the phenotypic changes observed in the injured and uninjured nerve that contribute to the generation of ectopic discharges (Bennett *et al.*, 1998). Ectopic discharges are massive spontaneous impulse discharges in mainly Aβ-fibres, but also Aδ-fibres, developing shortly after injury to nerves (Liu et al., 1999).

Long term changes include down regulation of the $Na_v1.8$ and $Na_v1.9$ in the injured nerves, but an up regulation of $Na_v1.8$ in uninjured nerves in surrounding tissue has been reported (Gold et al., 2003). The change in gene expression and thereby regulation in expression of ion channels, receptors etc. have been investigated by microarray with several hundreds of genes found to be up- or downregulated (Costigan *et al.*, 2002). Even though changes in gene expression have been reported for a number of the voltage gated sodium channels, none of the DRG tissue-specific transgenic mouse lines with deleted sodium channel(s) have shown any reduction in the development of neuropathic pain. Blockade of $Na_v1.8$ has however, shown a decrease in the development of neuropathic pain (Ekberg et al., 2006). The development of allodynia has been hypothesised to be mediated by increased activity in the Aβ-fibres. Touch-evoked activity has been shown to be increased in the superficial dorsal horn laminae suggesting that Aβ-fibres deliver non-nociceptive central information to the normally nociceptive laminae I+II (Bester *et al.*, 2000).

1.3.7 Important changes in gene expression due to neuropathic pain

The expression of several proteins, in particular $Na_v1.3$ and the Voltage Gated Calcium Channel (VGCC) subunit $\alpha_2\delta$-1 have been proven to change in neuropathic pain conditions.

1.3.7.1 $Na_v1.3$

Most of the voltage gated sodium channels expressed in the DRG become downregulated in neuropathic pain (Rogers et al., 2006). $Na_v1.3$ however, is almost absent in the adult mouse

DRG, but was shown to be upregulated after nerve injury, both in the DRG and CNS (Hains *et al.*, 2003;Waxman *et al.*, 1994). Later on, it was shown that down regulation of the $Na_v1.3$ using antisense oligonucleotides could reverse neuropathic pain (Lindia *et al.*, 2005). In contrast, when another group used antisense to knock down $Na_v1.3$ after having introduced neuropathic pain, no differences were observed between the antisense and the mismatch group (Lindia *et al.*, 2005). A separate analysis of the DRG tissue-specific knockout of the $Na_v1.3$ failed to show any differences in the development of neuropathic pain (Nassar *et al.*, 2006).

1.3.7.2 Voltage gated calcium channel subunit $\alpha_2\delta$-1

The VGCC $\alpha_2\delta$-1 subunit has been shown to be involved in the regulation of neuropathic pain (Newton *et al.*, 2001;Luo *et al.*, 2001). Following peripheral nerve injury, using several neuropathic pain models, two groups demonstrated an upregulation of both $\alpha_2\delta$ mRNA and protein expression in small and medium diameter DRG neurons. The upregulation was highest in the DRG, but also observed in the dorsal horn of the spinal cord. Further, Chun-Ying Li *et al.* used intrathecal administration of antisense oligonucleotides to knock down the $\alpha_2\delta$-1 in nerve-injured rats, and reported a reversal of the expected upregulation of $\alpha_2\delta$-1 in both DRG and spinal cord. It was apparent that antisense was involved in a reversal of mechanical allodynia to some degree, but the authors reported that the antisense treatment resulted in an incomplete knock down and large variations between treated animals were observed (Li *et al.*, 2004). From a pharmacological perspective, the anticonvulsant Gabapentin/Pregabalin binds to $\alpha_2\delta$-1 and has since then shown a better than existing drug profile for the treatment of neuropathic pain (Gilron, 2007).

1.3.7.3 Bradykinin B1

The Bradykinin B1 receptor is induced as a consequence of inflammation and plays an important role in inflammatory nociception. Recent studies have shown that B1 expression is upregulated in DRG after nerve injury (Levy and Zochodne, 2000), suggesting a role for B1 in the development of neuropathic pain. Work by Ferreira et al. 2005 showed a significant resistance to the mechanical allodynia and thermal hyperalgesia normally

observed after nerve injury. Further, using a B1 receptor antagonist made it possible to reverse the hypersensitivity observed in wild-type mice with nerve injury induced by partial sciatic nerve ligation (Ferreira *et al.*, 2005;Levy and Zochodne, 2000).

1.3.7.4 Microglia/BDNF

A publication from Watkins et al. reported that microglia, a type of glia cells, play a role in the development of pathological pain (Watkins et al., 2001) and in particular in neuropathic pain a robust change was seen (Griffin *et al.*, 2007). A blocker of microglial activation, minocycline, was shown to either prevent or delay the development of different models of neuropathic pain (Raghavendra et al., 2003). BDNF has shown to play a crucial role in the communication between the microglia and the neuron. A study showed that ATP activated microglia caused a shift in the GABA-A mediated inhibition to excitation in the spinal dorsal horn superficial neurons resulting in the release of BDNF. Using siRNA to block the release of BDNF from ATP activated microglia, suppressed both the current shift and allodynia. Similarly, blockage of the receptor for BDNF (TrkB) was shown to prevent both current shift and allodynia suggesting that BDNF is the main signalling molecule released from activated microglia contributing to allodynia after nerve injury. (Coull et al., 2005).

1.3.8 Dorsal horn termination after neuropathic pain

The central termini of sensory neurons, at the point where they synapse onto the secondary neurons in the spinal dorsal horn, exhibit numerous changes after nerve injury. It has been claimed that A-fibres, under normal circumstances terminating in lamina I and III-VI, injury can sprout into lamina II after nerve injury (Woolf et al., 1992). As described in section 1.2 lamina II normally receives information predominantly from C-fibres and the theory suggests that the sprouting may be one of the causes for the development of allodynia, one of the characteristics of neuropathic pain. The sprouting of A-fibres has also been shown not to be permanent and after a period of months the previously sprouted A-fibres are withdrawn from lamina II (Woolf et al., 1995).

1.4 CENTRAL PROCESSING OF NOCICEPTION

As described, primary neurons synapse onto secondary neurons, from where the nociceptive information is carried through to the sensory cortex. The main neurotransmitter released after moderate stimulation of the primary neurons is glutamate, which generates a fast synaptic response in the second order neurons mediated by the ionotropic N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and Kainate receptors as well as the G-protein coupled glutamate receptors. More intense nociceptive stimuli trigger the release of several neuropeptides such as SP, CGRP, H^+, ATP and BDNF into the synapse cleft.

The nociceptive signals pass through two different pathways to the brain. These pathways are referred to as the lateral polysynaptic pathway and the medial polysynaptic pathway. In the lateral pathway the signals reach the brain regions via the lateral thalamic nuclei. This pathway encodes the sensory discriminative properties of painful stimuli, which are perceived in the primary and secondary somatosensory cortices as pain. Only then are they localised to a specific region of the body. Signals transmitted through the medial thalamic nuclei reach the prefrontal and anterior cingulated cortices of the brain. This pathway is involved in encoding the motivational affective properties of pain stimuli, and here the stimuli are recognised as unpleasant and defence responses are mediated.

1.5 VOLTAGE GATED SODIUM CHANNELS

Voltage Gated Sodium Channels (VGSC) are involved in modulating the resting membrane potential (Herzog et al., 2001)) and sub-threshold oscillations, and in the generation and conduction of action potentials. They can be divided pharmacologically into two groups depending on their sensitivity to the guanidinium compound Tetrodotoxin (TTX), found on puffer fish '*Fugu*' where it is produced by bacteria. The VGSCs consist of the pore forming α-subunit and one or more auxiliary β-subunits (see fig.4). The β-subunits stabilise the channel in the plasma membrane and modulate the properties, but are not essential for the channel function (Isom et al., 1994). In total ten different isoforms of the α-subunit ($Na_v1.1$-1.9+Na_X) and five β-subunits (β_1-β_4+β_{1a}) have been identified and cloned. The TTX-sensitive $Na_v1.1$, Na_v 1.2, and Na_v 1.6 are all expressed in the CNS and primary

sensory neurons. Another TTX-sensitive sodium channel, $Na_v1.7$ is expressed in sympathetic neurons, Schwann cells and primary sensory neurons (Toledo-Aral *et al.*, 1997;Sangameswaran *et al.*, 1997). Finally, $Na_v1.8$ and $Na_v1.9$ are expressed exclusively in sensory neurons and are resistant to TTX (Dib-Hajj et al., 1998).

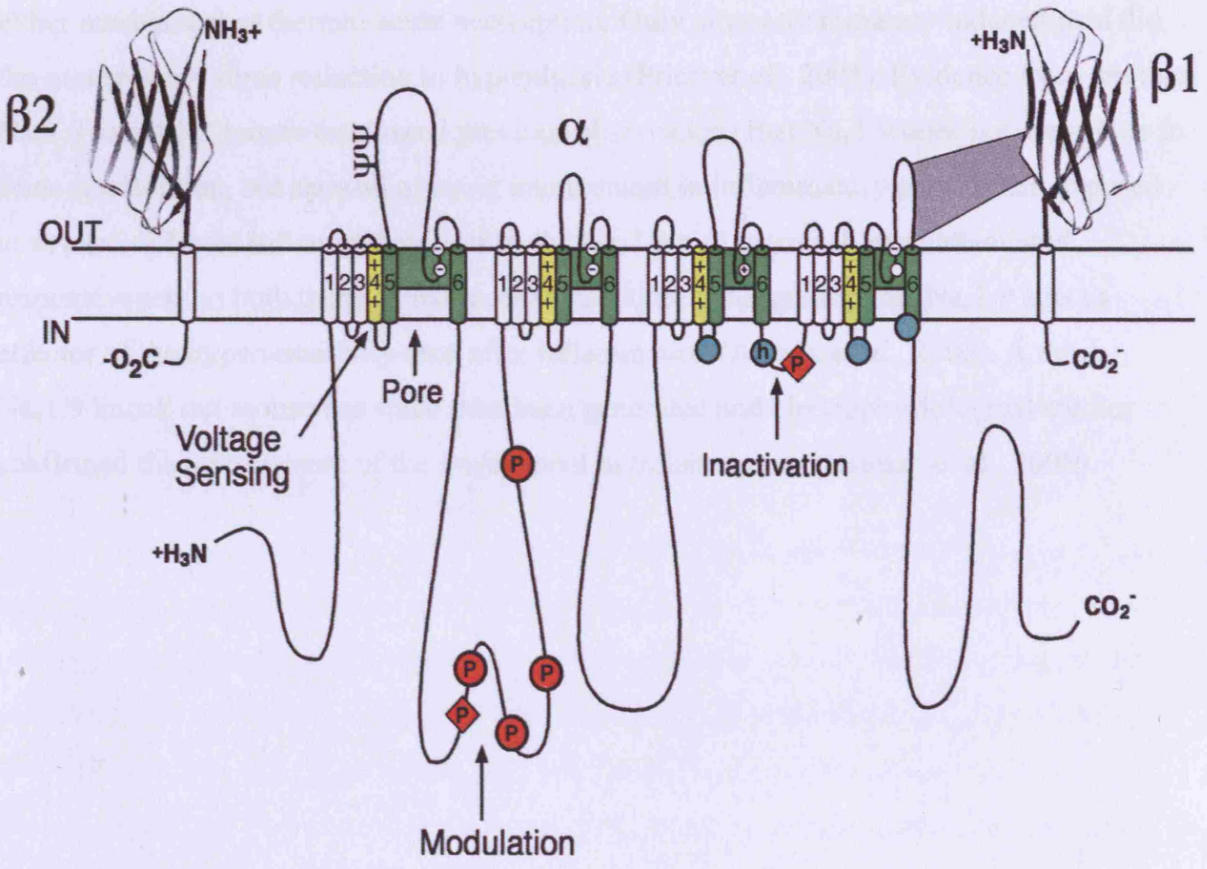

Figure 4. Structure of the Voltage Gated Sodium Channel. From (Scheuer and Catterall, 2006)

The role for $Na_v1.8$ in nociception was based on its exclusive expression in over 85% of the small sensory neurons (nociceptors) (Akopian *et al.*, 1996;Okuse *et al.*, 1997). In the $Na_v1.8$ null mouse, a phenotype to noxious mechanical and some differences to thermal and inflammatory stimulation were found (Akopian *et al.*, 1999).

A double KO of both $Na_v1.7$ and $Na_v1.8$ showed no difference in the development of neuropathic pain, but a very significant reduction in the second phase of the formalin model, slightly stronger than the reduction seen with the $Na_v1.7$ KO alone (Nassar *et al.*, 2005). Interestingly, when exposed to thermal stimulation the double KO showed a greater latency in response compared with both the $Na_v1.8$ KO and the $Na_v1.7$ KO. This suggests

that each channel has an individual role in pain, but possible a combined role which is not completely understood.

$Na_v1.9$ is also expressed exclusively in nociceptors in the DRG, but data from a transgenic mouse line with a disrupted $Na_v1.9$ channel showed little involvement of the channel in either mechanical or thermal acute nociception. Only after inflammatory induced pain did the mouse show some reduction in hyperalgesia (Priest *et al.*, 2005). Evidence from another $Na_v1.9$ knockout mouse confirmed previous observations that $Na_v1.9$ does not contribute to acute nociception, but showed a strong involvement in inflammatory pain. When exposed to several different inflammatory agents, the $Na_v1.9$ null mouse showed attenuated responsiveness to both thermal and mechanical stimuli, suggesting that $Na_v1.9$ acts as effector of the hypersensitivity seen after inflammation (Amaya *et al.*, 2006). A third $Na_v1.9$ knock out mouse has since then been generated and electrophysiological studies confirmed the involvement of the ion channel in inflammation (Ostman et al., 2008).

2 EXPERIMENTAL APPROACHES

2.1.1 Electrophysiology

Electrophysiological recording of either cells or tissue preparations is a well-established method enabling investigation of ion-channel related electrical current over the cell membrane. Historically, sophisticated methods have been developed making it possible to record from WDR neurons. The patch clamping technique is one of the most recognised methods for scientific investigations of cellular currents and many different techniques have emerged to test the effect upon current, after physical stimulation of the cell such as heat, pressure etc. Also, recordings from WDR neurons make it possible to measure central input after peripheral stimulation.

2.1.2 Extracellular electrophysiology

Using electrophysiological recording from single unit WDR neurons makes it possible to investigate the modulations in nociceptive pathways at the dorsal horn level after stimulation of the periphery. This technique is mostly used on rats, but Dickenson et al have modified it for use in mice (Urch and Dickenson, 2003;Foulkes *et al.*, 2006). After the electrode has been placed (extracellularly) in the WDR neuron in a deeply anaesthetised animal, recordings are made after electrical, mechanical or thermal stimulation of varying magnitude to the intact hind paw.

The latency relative to initial stimulus makes it possible to distinguish between A- and C-fibre mediated responses. A whole range of responses is normally recorded and used to characterise the properties at dorsal horn level, examples of which include the analysis of two different DRG tissue-specific knockouts, $Na_v1.7$ (Nassar *et al.*, 2004) and Annexin 2 light chain P11 (Foulkes *et al.*, 2006).

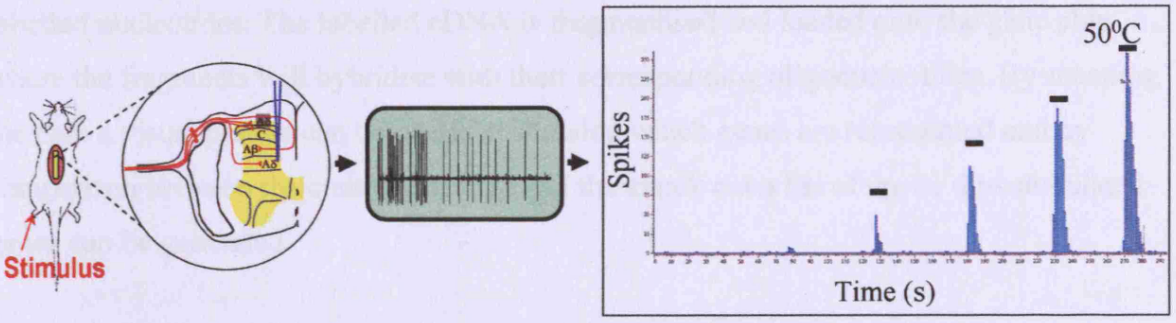

Figure 5. Single unit electrophysiology.
The electrical information generated from stimulation of the peripheral nerves is identified by the electrode and amplified.

2.1.3 Patch clamp electrophysiology

The patch clamp technique makes it possible to record electrophysiological characteristics from either small numbers of ion channels located in the cell membrane or from the whole cell. A small glass pipette with a very narrow diameter is attached to the cell with light suction. The solution in the pipette is prepared to match the intracellular solution as closely as possible. An electrode in the pipette conducts the electrical changes in the membrane potential after changes to the extracellular solution or physical stimuli. If the cell membrane is broken the recordings will be from the whole cell giving a much larger current. A review of different patch clamp techniques is described by (Neher, 1992).

2.2 MICROARRAY

Functional genomic studies, where mRNA expression is analysed under defined conditions (e.g. healthy and diseased), allow us to compare different sets of genes under given conditions. Microarray is a tool to measure the expression level of thousands of genes by determining the amount of mRNA that is present e.g. differences in gene expression between KO and WT.

The Affymetrix gene chip is manufactured by using photolithography combined with combinatorial chemistry to produce short strands of oligonucleotides, representing the mouse genome, synthesised onto a silica slide.

The method works in principle by making labelled cRNA from cDNA, synthesised from mRNA extracted from the desired tissue using poly-T-primers, reverse transcriptase and

labelled nucleotides. The labelled cDNA is fragmentised and loaded onto the gene chip where the fragments will hybridise with their corresponding oligonucleotides. By scanning the chip a visual picture can be obtained showing which genes are represented and by comparison between the control-baseline and the knock out a list of up- or downregulated genes can be generated.

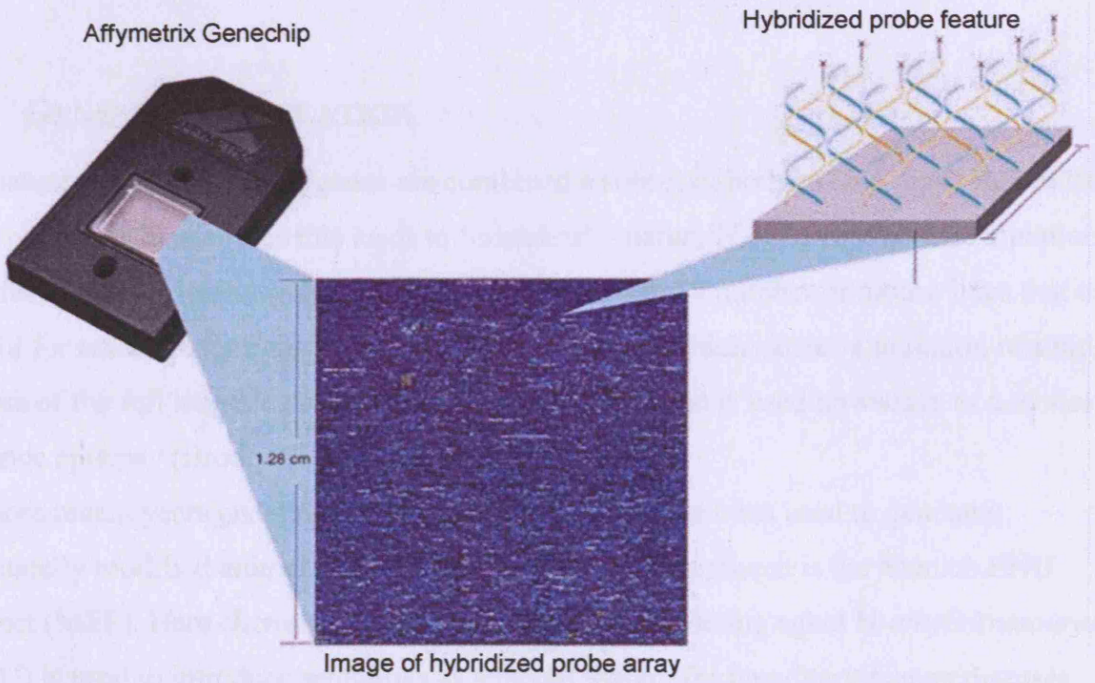

Figure 6. The Gene chip technology.
Thousands of short sequence oligonucleotides (probes) are representing different genes. The hybridization of the sample to the probes can be detected by fluorescence.

Microarray technology has been used increasingly over the last 5-10 years. Among the first microarray data to be published was the measurement of differences in gene expression from 45 *Arabidopsis thaliana* genes (Schena *et al.*, 1995). Since then the availability and affordability together with reliability has promoted the use of microarray to one of the most commonly used methods for the study of gene expression. There remains a dearth of published microarray studies related to pain, but among some of the more recent papers is a comparison of gene regulation at spinal horn level between three neuropathic pain models (Griffin *et al.*, 2007) and another comparison at dorsal horn level of the regulation of genes after CFA induced inflammation (Geranton *et al.*, 2007).

One of the things microarray will not reveal is any difference in the translation of mRNA into functional protein. Analysis by Western blot would be able to show changes in protein levels.
Another important thing is the possibility of false positive genes. Even though more than half the probes on the chip is controls a risk is always there and confirmation using qRT-PCR is required to validate the microarray data.

2.3 GENETIC MANIPULATION

By nature, when two sets of genes are combined a subset of both is, then replicated in the new organism. Sometimes this leads to 'mistakes' – naturally occurring genetic mutations - and fortunately scientists have over the years, discovered a number of mouse lines that are useful for research. One example is the "ducky mouse" which carries a mutation resulting in loss of the full length calcium channel subunit $\alpha_2\delta$-2 and is used nowadays as a model for absence epilepsy (Brodbeck et al., 2002).
In more recent years genetic manipulation techniques have been used to generate genetically modified animals and a more systematically approach is the Munich ENU Project (MEP). Here chemical mutagenesis using the alkylating agent N-*ethylnitrosourea* (ENU) is used to introduce mutations as a model system for hereditary human diseases.

2.3.1 RNA interference

The use of antisense is thought to be able to 'switch off' genes and block protein expression. By injecting RNA sequence complementary to mRNA transcribed from the gene of interest, the antisense RNA would hybridize to the mRNA and block translation. Difficulties with delivery, stability and specificity have limited the use of antisense, particularly *in vivo*.

A more recent discovery from work on C.elegans (Montgomery et al., 1998) investigated the effect of short double stranded RNA sequences and how they could trigger the natural degradation of homologous mRNA. Since then, it has been demonstrated that double stranded RNA of more than 30 nucleotides initiates an interferon based inflammatory response that causes unspecific translational suppression involving the dsRNA RNAse III

enzyme DICER (Elbashir et al., 2001). Today, most studies use 21-23 nucleotide synthetically RNA duplexes. The duplex assembles with a multiprotein nuclease complex, RNA-Induced-Silencing-Complex (RISC), which unwinds the dsRNAs and degrades target mRNAs homologous to the single-stranded siRNA in a sequence-specific manner.

2.3.2 Targeted gene deletion by homologous recombination

Reliability and reproducibility are very important in any research, and the use of transgenic animals can be an immensely powerful tool, both reliable and reproducible, to investigate the function of a particular protein. Since its first use in the early eighties, reliability was a problem due to the 'random insertion' after pronuclear injection into fertilized single cell mouse embryos (Palmiter et al., 1982). There was no control over insertion site or number of copies, so each transgenic line generated could have its own pattern, making it difficult to distinguish the desired pattern (Feng et al., 2000).

Using homologous recombination to 'knock in' the transgene into a specific locus increased the reliability of the procedure, and with the use of tissue-specific recombinase systems early embryonic lethality could also be prevented by restricting the gene deletion to the tissue expressing the enzyme. Nonetheless, some issues remained as the enzyme must be driven by a specific promoter, which means the gene deletion would only happen when this promoter was active. To resolve this problem, several drug-inducible and tissue-specific Cre mouse lines were developed using different drugs to switch on the recombinase enzyme (Feil et al., 1997).

2.3.2.1 The Recombination system Cre/*loxP*

This system consists of the **C**yclization **R**ecombination protein (**Cre**) of the P1 bacteriophage and its short DNA recognition sequence *loxP*: ATAACTTCGTATAATGTATGCTATACGAAGTTAT. The recombination protein Cre catalyses the recombination of the sequence between the *loxP* sites, depending on the orientation of the sites. If the *loxP* sites are placed in opposite directions, the sequence between the *loxP* sites will be inverted by Cre. If sitting in the same direction, the flanked sequence will be excised.

Two different transgenic mouse lines are needed to use this system. One mouse expresses the recombination protein Cre and the other has been modified so *loxP* sites are flanking the gene of interest. In general, a plasmid has to be constructed that will integrate into the genome when transfected into embryonic stem cells.
For the floxed mouse, the construct contains loxP sites flanking the sequence of interest and next to them sequences homologous to the genomic sequence promote homologous recombination, the event where DNA from donor and host is exchanged. For positive selection a neomycin cassette is inserted between the loxP site and the 3'arm. For negative selection, a thymidine kinase cassette is inserted downstream of the 3'arm. This is useful to detect random insertion errors occurring in the homologous recombination.
The Cre-construct contains the chosen promoter followed by the coding sequence for Cre starting with ATG. The sequences of homology and selection cassettes are as for the floxed construct.

After successful recombination has been confirmed the stem cells are introduced into blastocysts and inserted into "pseudo-pregnant" mice for gestation. The Cre/*loxP* system is now a well established system and several reviews exist describing the development, mechanisms and possibilities using the recombinase systems (Nagy, 2000) and (Lewandoski, 2001)

2.3.2.2 Flp-FRT system

This system is based on the budding yeast *Saccharomyces cerevisiae* and works like Cre as a site specific recombinase. The Flp recognition target FRT repeats which have similar size properties as the *loxP* sites with the following sequence:
GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC.
Due to its origin in yeast, the Flp is optimal at 30°C and has been subsequently modified to optimise its performance at 37°C.

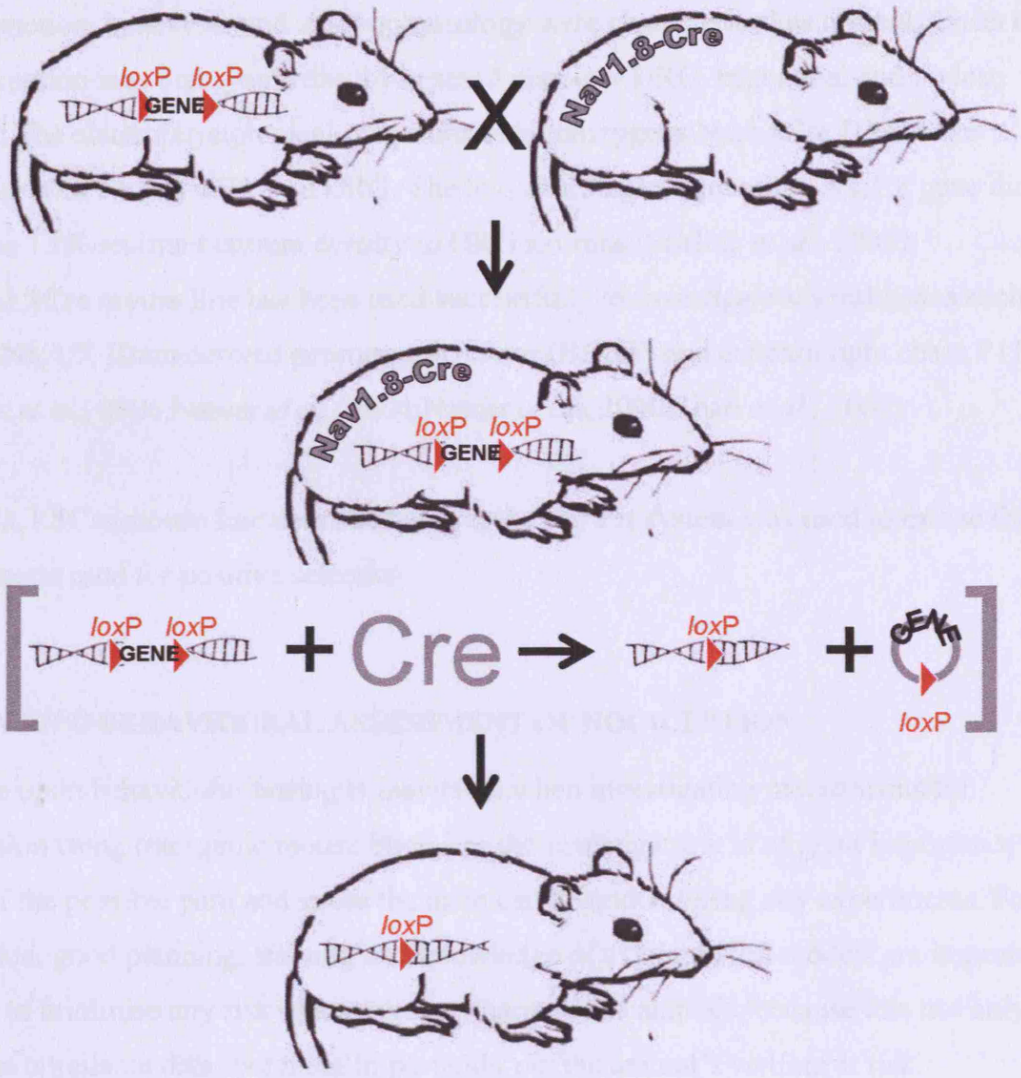

Figure 7. The Cre/*loxP* system.
The Cre expressing mouse is crossed with a floxed mouse to generate the tissue-specific KO mouse. Cre will recognise the *loxP* sites and delete the sequence flanked by the sites.

2.3.2.3 The $Na_v1.8$Cre mouse line

A Cre recombinase mouse line was generated by Stirling et al. linking the expression of Cre to the $Na_v1.8$ promoter (Stirling *et al.*, 2005). The Voltage Gated Sodium Channel $Na_v1.8$ was identified by Akopian et al. and its distribution limited to a subset of sensory neurons of which >85% are nociceptors (Akopian *et al.*, 1999;Djouhri *et al.*, 2003). Knock-In technique was used place the Cre-recombinase coding sequence at the ATG initiator of the $Na_v1.8$. The expression pattern of Cre was confirmed to mimic the pattern of $Na_v1.8$ and

motor function, behaviour and electrophysiology were characterised as normal. Onset of Cre expression is at embryonic day 14 in small diameter DRG, trigeminal and nodose neurons. The electrophysiological properties of heterozygous $Na_v1.8$Cre DRG were compared with $Na_v1.8$ wild type DRG. The loss of a single copy of the $Na_v1.8$ gene did not affect the TTX-resistant current density in DRG neurons (Stirling *et al.*, 2005).
The $Na_v1.8$Cre mouse line has been used successfully to investigate several genes such as $Na_v1.3$, $Na_v1.7$, Brain derived neurotrophic factor (BDNF) and annexin light chain P11 (Foulkes *et al.*, 2006;Nassar *et al.*, 2004;Nassar *et al.*, 2006;Zhao *et al.*, 2006)

In the $Na_v1.8$Cre mouse line described above, the Flp-Frt system was used to excise the Neo cassette used for positive selection.

2.4 *IN VIVO* BEHAVIOURAL ASSESSMENT OF NOCICEPTION

Reliance upon behavioural testing is inevitable when investigating mechanisms for nociception using transgenic mouse lines. For the investigator it is of great importance to be aware of the possible pain and stress the mice can be under during any experiments. For this reason, good planning, training and knowledge of available test models are important in order to minimise any risk of unnecessary harm to the animals, because this not only results in unreliable data, but more importantly put the animal's welfare at risk.
A general overview of animal behaviour is described in (Hogan, 2002).

2.4.1 Mechanical

The two preferred methods of mechanical testing to mimic innocuous and noxious mechanical stimuli are von Frey filaments and the Randall Selitto apparatus. A number of other methods have been reported and will be discussed below.

2.4.1.1 von Frey filaments

Von Frey testing is one of the most widely used methods by which to assess mechanical response threshold. Calibrated nylon filaments deliver a graded mechanical force to the hind paw of the mouse. The filament is applied for around 2-3 seconds and a positive response is recorded if the mouse lifts its paw during application.

One way the experiment can be performed is by applying a set of hair filaments, ranging from 0.07g to 1.4g, 5 times each and recording any positive responses, as described by Fuchs et al (Fuchs et al., 1999). The analysis is based on the percentage of positive responses for each hair and the 50% threshold can be determined.
Another method is the up-and-down method described by Chaplan et al. which benefits from using fewer applications of filaments, but still calculates the 50% threshold. The filament expected to give a positive response 50 percent of the time is applied. This application is followed by a filament of weaker strength if the mouse did respond or of stronger rigidity if it did not respond. This is repeated several times either side of the 50% threshold. The pattern of positive responses together with the force of the last hair applied is used to calculate the 50% threshold (Chaplan *et al.*, 1994).

2.4.1.2 Randall Selitto apparatus

The Randal Selitto test was developed by Randall and Selitto in 1957 and originally developed and used on rat paws, but has since then been modified to use on mouse tails (Takesue et al., 1969).
When using the Randall Selitto apparatus an increasing mechanical pressure from a blunt probe is applied to the tail of the mouse until a withdrawal response is registered and the final weight is recorded. When using the test on rats the experimenter has to train the rats so the instrument can be applied to the paw. When using on mice, a Perspex glass restrainer is used to access the tail.

Using von Frey, a blunt thin filament is applied. The response is believed to be due to touch sensitive neurons. It is not a noxious stimulus, but an irritant sufficient to evoke a response. The Randal Selitto apparatus applies an increasing pressure from a $3mm^2$ blunt probe activating high-threshold mechanoreceptors in the C-fibres and is considered to be a response to a noxious insult (Hogan, 2002).

2.4.2 Thermal

A host of different thermal stimulation methods exist and five are described briefly in the following paragraphs.

2.4.2.1 Hargreaves' test

The Hargreaves instrument allows the mouse to settle in a small chamber on top of a glass surface, before thermal responses are tested using a focused light beam applied to the hind paw through the glass plate. The latency in seconds until the mouse first responds to the heat is monitored. The test was described by (Hargreaves et al., 1988) and is considered to be a spinal reflex due to the fast paw withdrawal.

2.4.2.2 Hot plate

Using the hot plate test, the mouse is placed on a hot plate between 50-55°C until any licking, biting or flinching in the hind paws is observed. The response is considered a supra-spinal response involving higher centres (Jensen and Yaksh, 1984) and was first described by Woolfe and MacDonald (WOOLFE, 1943).

The different techniques used in the two thermal tests evoke a different type of response in the mouse. The Hot plate exposes the paw (and mouse) to a constant thermal stimulus, whereas the Hargreaves is a radiant heat source only applied to the paw. As noted above this results in different patterns of withdrawal.

2.4.2.3 Tail-flick test

This test is today performed by exposing the tail to a radiant heat source and measuring the latency in seconds before the tail flicks away from the heat. The mouse is placed on a base with its tail placed on top of a focused beam. When the mouse has settled the beam is activated and delivers a strong noxious thermal stimulus. Historically this test had been performed by immersing the tail in warm or cold water.

2.4.2.4 Cold pain

Few models for the investigation of noxious cold have been described. Among them, the most typical way is to use a temperature-controlled plate cooled to 0°C. After the mouse is placed on the plate, lifts of the paws are counted for 5 minutes. This test is repeated twice with at least one hour in between (Zimmermann *et al.*, 2007).

Another test, the temperature preference test, uses the same principles, but has two plates in conjunction with each other and is probably a non-noxious test. Using this set-up two different temperatures can be programmed and if the mouse does not respond to the cold, the time spent on each plate should be the same (Colburn *et al.*, 2007). Finally, the application of a volatile liquid, such as acetone, undergoes an endothermic reaction in the change of phase from liquid to gas. The time the mouse spends licking and biting the affected paw is measured.

2.4.3 Inflammatory models

Inflammatory models can be wide-ranging depending on the compound inducing the response. Formalin is a very short-lasting model with the response ending after less than an hour. The response to Carrageenan lasts for several hours whereas CFA gives rise to a long-lasting inflammatory response that can be followed for days. Specific receptors or ion channels can be tested by using different compounds such as Substance P (NK-1), Capsaicin ($TrpV_1$), NGF (TrkA) etc.

2.4.3.1 Formalin

Injecting 5% formalin into the plantar surface of the paw results in tissue damage and a typical and widely described behavioural pattern in the mouse. The response can last up to one hour and is divided into two phases: the first 5 minutes are considered direct chemical stimulation of the free nerve endings; the response from 10-60 minutes is considered inflammatory as modulators are released in the affected tissue.

2.4.3.2 Complete Freund's Adjuvant (CFA)

Complete Freund's Adjuvant consists of dried and inactivated *Mycobacterium butyricum* emulsified in mineral oil, inducing an inflammatory response. Compared with injection of Formalin, CFA, when injected into the plantar surface of the hind paw, generates a long term inflammatory response resulting in both mechanical and thermal hyperalgesia tested using von Frey filaments and Hargreaves' instrument respectively. The model, described

for use in rats by (Millan et al., 1988) results in an inflammatory response developing within 24 hours and lasting for longer than one week.

2.4.3.3 Carrageenan

Carrageenans are a complex group of polysaccharides, extracted from red seaweed, made up of repeating galactose-related monomers, which form thermo reversible gels. There are three main types – lambda (λ), kappa (κ) and iota (ι) – each with different characteristics. λ-carrageenan does not form a gel at room temperature and can therefore be used for injection, resulting in a reproducible inflammatory response that is maximal 3-5 hours following injection (WINTER et al., 1962). Thermal hyperalgesia is measured using Hargreaves' instrument.

2.4.3.4 Nerve Growth Factor

Nerve Growth Factor expression is upregulated due to inflammation (Woolf et al., 1994) and can be assessed by injection of NGF direct activation of the TrkA receptor, expressed in nociceptors (Fang et al., 2005). Injection of NGF is widely used to further characterise the inflammatory response. The hyperalgesic response after NGF injections is tested using thermal stimulation.

2.4.3.5 Capsaicin

$TrpV_1$ is activated by Capsaicin and specific responses to capsaicin can be performed by injecting Capsaicin (0.5-2.5μg/paw) into the plantar surface of the paw and monitoring the licking/biting behaviour for up to 5 minutes (Dhaka *et al.*, 2007).

2.4.4 Spontaneous pain

Models for spontaneous pain are not very well established, but recently Djouri et al. described a model for rats using injection of CFA (Djouhri et al., 2006). After injecting CFA, both in the paw and to the side of the knee joint, behavioural observations were performed on day 1, 2, 3 and 7. The spontaneous pain was reported as the cumulative time

the rats spend having spontaneous foot lifting over two periods of 5 minutes. This model of spontaneous pain has not previously been reported for use in mice.

2.4.5 Neuropathic pain models

There exist a variety of animal models for neuropathic pain that interfere with the sciatic nerve and as such attempt to be analogous to human neuropathic pain. Fig.8 gives an overview of some of the models.

2.4.5.1 Chung

The Chung models, described in rats by Kim and Chung (Kim and Chung, 1992) are two different models of ligation of the spinal nerve innervating the hind paw. The first model involved tight ligations of L5 and L6; the second only a tight ligation of L5. Substantial innervation from the remaining spinal neurons to the hind paw would still exist, allowing testing for hyperalgesia. Behavioural analysis revealed development of mechanical hyperalgesia lasting many weeks with thermal hyperalgesia also developing, but more short-lived than the mechanical. Since then, Honore et al. (2000) have tested the second model on mice and evidence suggests that they develop the same characteristics as those observed in rats (Honore et al., 2000).

2.4.5.2 Seltzer (PSNL)

Partial Sciatic Nerve Ligation (PSNL) is a ligation of ⅓-½ of the sciatic nerve and was described by Seltzer et al. in rats in 1990 (Seltzer et al., 1990) and modified to mice by Malmberg and Basbaum in 1998. The surgery results in a reproducible syndrome in the mouse, where a decrease in thermal and mechanical nociceptive thresholds is observed as well as prolonged changes in neurotransmitter and receptor expression (Malmberg and Basbaum, 1998).

2.4.5.3 Other neuropathic pain models

The Chronic Constriction Injury (CCI) model was developed and described by Bennett and Xie involving four loose ligations around the sciatic nerve (Bennett and Xie, 1988).

The spared nerve injury model (SNI) is based on axotomisation of the tibial and peroneal nerves sparing the sural nerve (Decosterd and Woolf, 2000).

For all models using ligations it is important to be consistent with the tightness of the ligations. For SNI and CCI and PSNL the surgery is simple, but to reproduce the thickness of the sciatic nerve ligated can be difficult due to the size. With the CCI the tightness of the ligations also has to be reproducible as up to four of them are placed around the sciatic nerve. Chung (SNL) on the other hand, is very reproducible, but is much more invasive and the surgery itself is more delicate and can take up to 30 minutes per animal.

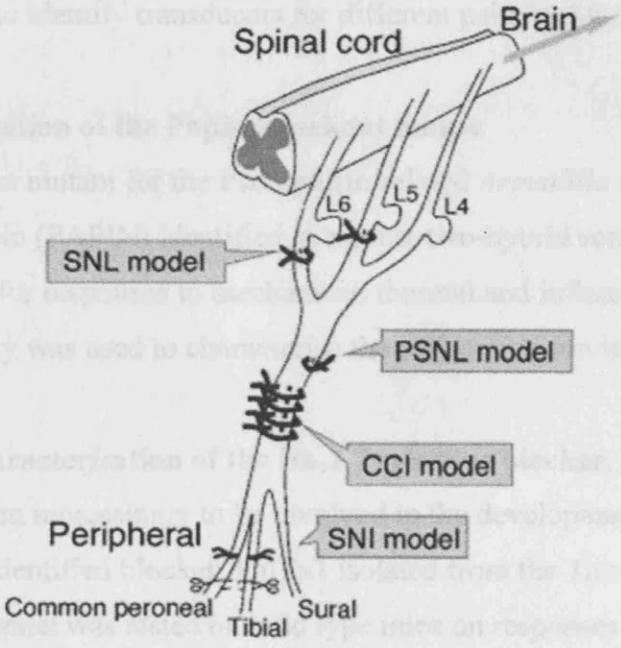

Figure 8. Models of neuropathic pain.
SNL; Sciatic Nerve Ligation (Chung),
PSNL; Partial Sciatic Nerve Ligation (Seltzer)
CCI; Chronic Constriction Injury
SNI; Spared Nerve Injury.
From (Ueda, 2006)

PROJECT OUTLINE

A brief introduction to each part of my project will be included for the respective chapters.

The main parts include:

Tissue-specific deletion of $Na_v1.8$-expressing sensory neurons

Using the tissue-specific $Na_v1.8$Cre mouse line crossed with the Diphtheria toxin fragment-A floxed mouse line, a nociceptor specific transgenic mouse line with all $Na_v1.8$-expressing neurons deleted was generated making investigations into the role of $Na_v1.8$-expressing neurons possible. As described above, behavioural assessment, immunohistochemical description and single unit electrophysiology was used to characterise the mouse. Finally microarray was used to identify transducers for different pain modalities.

Behavioural investigation of the Papin knockout mouse

A knockout mouse line mutant for the Plakophilin-related *Armadillo* repeat Protein-Interacting PDZ protein (PAPIN) identified in a yeast-two-hybrid screening using $Na_v1.8$ as bait was investigated for responses to mechanical, thermal and inflammatory stimulation. Immunohistochemistry was used to characterise the cell expression in the DRG tissue.

Pharmacological characterisation of the $Na_v1.7$ selective blocker, PhlTx1

$Na_v1.7$ has been proven increasingly to be involved in the development of several pain modalities. A recent identified blocker, PhlTx1 isolated from the Tarantula spider, specific for the $Na_v1.7$ ion channel was tested on wild type mice on responses to mechanical, thermal and inflammatory responses and after the introduction of neuropathic pain.

3 MATERIALS AND METHODS

3.1 MOLECULAR BIOLOGY

3.1.1 Genotyping

Mice of at least 4 weeks of age were marked using an ear puncher. The tissue samples from the ear were used for genotyping. Tissue samples from the tail was in some cases obtained for genotyping as described below.

3.1.1.1 DNA extraction

From tail sample:
2-3 mm of tail was snipped from each animal according to the Animals (Scientific Procedures) Act 1986. The tissue was incubated in a microfuge tube holding 500 µl of lysisbuffer for 6-12 hours at 55°C.

DNA Lysis buffer
100 mM Tris-HCl (pH 8.0)
5 mM EDTA
0.2% SDS
200 mM NaCl
(Stored at RT)
To each tail sample Proteinase K (PK) was added in 1:200

After incubation the solution was vortexed and any undigested tissue was isolated by spinning at 14.000 rpm for 5 min before the supernatant was transferred to a new microfuge tube.
One volume of isopropanol was added and the tube inverted to precipitate the DNA, followed by centrifugation at 14.000 rpm for 15 min.
The supernatant was discharged and the pellet was washed in 500 µl 70% ethanol twice. After removing the supernatant the pellet was air dried for 2-5 min before the DNA was resuspended in 200 µl TE buffer.
The PK was inactivated for 15 min at 65°C.

If further purification was needed, the supernatant from the extraction was mixed with phenol/chloroform, vortexed and spun for 2 min at 14.000 rpm. The supernatant was transferred to a new tube and 1 volume of isopropanol was added. After inverting the tube a few times, the tube was spun for 5 min at 14.000 rpm. The cleaning of the DNA was performed as above.

From ear tissue sample:
Alternatively, DNA from a small piece of ear tissue was extracted using the following protocol:

3.60 ml	10X GB
0.72 ml	25% TritonX-100
0.36 ml	b-mecaptoethamol
31.32 ml	H_2O
36.00 ml	

For each 475 µl PCR lysis buffer, 1 µl of 19.7 mg/ml Proteinase K (Roche 03 115n844) was added before use.

10X GB (store at -20 degree)

4.47 ml	1.5M Tris pH8.8
1.66 ml	1.0M $(NH_4)_2SO_4$
0.67 ml	1.0M $MgCl_2$
3.20 ml	H_2O
10.00 ml	

The samples were incubated at 55°C for 1 hour followed by 95° for 5 min to inactivate the Protenaise K. The samples were vortexed briefly and kept at -20°C.

3.1.1.2 PCR

Polymerase chain reactions were used to genotype the DNA from each animal.

DTA-control (no floxed band)	Tm	Product
S 5'-AAGTCGCTCTGAGTTGTTAT-3'	58°C	600 bp
AS 5'-GGAGCGGGAGAAATGGATATG-3'		

DTA-floxed (floxed band)	Tm	Product
S 5'-AAGTCGCTCTGAGTTGTTAT-3'	59°C	300 bp
AS 5'-GCGAAGAGTTTGTCCTCAACC-3		

Cre recombinase	Tm	Product
S 5'-AAATGTTGCTGGATAGTTTTTA-3'	64°C	450 bp
AS 5'-AAATTTGCCTGCATTACCGGTCGA-3		

PAPIN-WT	Tm	Product
S 5'-CAAGAGTCACTTATCTTTGTAAGACCTCTA-3'	58°C	420 bp
AS 5'-ACTTATGGAACAGAATATAAAAGGCAAG-3'		

PAPIN-KO	Tm	Product
S 5'-CAAGAGTCACTTATCTTTGTAAGACCTCTA-3'	58°C	570 bp
AS 5'-GGATAAGTATGACATCATCAAGGAAAC-3'		

In general the following conditions for the PCR reactions were used:

0.5 µg template DNA
2.5 µl 10 × PCR Buffer
1.5 µl $MgCl_2$ (25 mM)
1.0 µl dNTP (5 mM)
1.0 µl of each primer (50 µM)
$^{dd}H_2O$ up to 24.7 µl
0.3 µl Taq polymerase
Vol_{tot}: 25µl

3.1.1.3 PCR protocol:

Denaturation at 94°C for 3 min.
Denaturation at 94°C for 30 sec.
Annealing at 58-66°C for 40-70 sec (Tm).
Extension of the product at 72°C for 1-2 min.
Repeating step 2-4 29-34 times
Keeping the PCR product at 10°C ∞.

3.1.2 Gel electrophoresis

For separation of RNA or DNA bands by size, agarose gel electrophoresis was used 0.8-1.5 percent agarose gels, depending on the sizes of fragments, were prepared by dissolving agarose (Sigma, UK) in TAE 1X buffer 1XTAE (40mM Tris-acetate, 1mM EDTA). After cooling Ethidium bromide was added (0.5μg/ml) before the gel was loaded onto the gel tray and left to set. The samples were loaded into separate wells together with a 1-3 μl of loading buffer. From a power supply 80-120 volts were used to separate the bands. Bands were visualised using UV, UVP Biodoc-It™ Systems, and a picture printed from a thermal printer (Mitsubishi Video Copy Processing)

3.2 AFFYMETRIX MICROARRAY

3.2.1 RNA isolation

DRG from DTA-control and DTA CRE animals were dissected and kept in RNAlater (Qiagen, UK). From each animal one DRG was kept for immunohistochemistry control. The tissue was pooled in groups of two mice and the tissue homogenised using glass homogeniser cleaned with ddH2O followed by DEPC treated water (Ambion, UK) and finally once in 1 ml Buffer RLT. The tissue was kept on ice throughout the procedure. The tissue samples were passed through a QIA shredder column (Qiagen, UK) and spun down for 2 min at 14K rpm. The supernatant was transferred to a clean tube and 1 volume of 70% ethanol was added and mixed. The mixture was added to an RNeasy column and spun for 15 sec. at 14K rpm. Buffer RW1and twice Buffer RPE was added in turn, spun and the

flow-through added, followed by the next buffer was added. Finally the column was placed in a collection tube and the column spun for 1 min at 14K rpm. The column was then placed in another collection tube and RNase free water was added to the column membrane before being spun again at 14K rpm for 1 min.

The concentration and quality of the RNA was measured using Nanodrop (BiotechLab, UK) and a minigel (Experion, Bio-Rad) was run to check the 28s and 18s ratio. A 28s/18s ratio around 2 is considered very good, but less than 2 is also acceptable. Finally the sample was stored at -80°C.

3.2.2 First strand cDNA Synthesis

One-Cycle cDNA Synthesis kit (Affymetrix) was used for first and second strand cDNA synthesis.

The RNA eukaryotic sample (2μg), poly-A RNA controls and T7-oligo(dT) primer were mixed with RNase free water. The tubes were gently mixed and spun down for 5 sec. before being incubated for 10 min at 70°C. The tubes were cooled down and spun briefly again to collect the sample.

In parallel the First-Strand Master Mix was prepared. 7μl was added to each tube of RNA/poly-A/T7 mixture before the tubes were incubated for 2 min at 42°C.

1 μl of SuperscriptII was added and the contents of the tubes gently mixed before incubating for a further 60 min. at 42°C. The tubes were cooled to 4°C and spun to collect the sample and stored at 4°C for the second-strand cDNA synthesis.

3.2.3 Second-strand cDNA synthesis

The Second-strand Master Mix was prepared and added to each first-strand synthesis sample. The tubes were briefly spun before incubating for 2 hours at 16°C. T4 DNA Polymerase was added and incubated for 5 min further at 16°C. After incubation 0.5M EDTA was added and the sample was cleaned.

3.2.3.1 Cleanup of Double-Stranded cDNA

To each cDNA tube 600µl cDNA 'Binding Buffer' was added. The solutions were mixed and added to a cDNA Cleanup column placed in a collection tube. The column was spun down for 1 min at 10K rpm and the flow-through discharged.

The column was washed using cDNA Wash Buffer and spun for 1 min at 10K rpm followed by a further 5 minutes spun at 14K rpm. cDNA Eluation Buffer was added to the column membrane and collected by spinning the column at 14K rpm for 1 min.

3.2.3.2 *In vitro* transcription of Biotin-Labelled cRNA Synthesis

To the template cDNA a mixture of labelling buffer, labelling NTP mix and labelling enzyme mix (IVT Labelling Kit, Affymetrix) was added and gently mixed before incubating at 37°C for 16 hours. The labelled cRNA was stored at -80°C.

3.2.3.3 Clean-up of Biotin labelled cRNA

Gene chip Sample Cleanup Module (Affymetrix) was used for the cleanup of cDNA

The labelled cRNA was cleaned using Affymetrix Clean-up Module (Affymetrix, US). To each tube RNase free water, Binding buffer and ethanol were added, mixed, and the total transferred to a cleanup spin column. The column was spun briefly before wash buffer was added and followed by another brief spin.

The cRNA quality and concentration was checked using the Nanodrop (BiotechLab, UK) instrument.

3.2.3.4 Fragmentation

20µg of labelled cRNA was mixed with 8µl of 5x Fragmentation Buffer (Affymetrix Clean-Up module) the mixture was incubated for 35 min at 94°C.

The fragmentation was checked on a mini gel (Experion, Bio-Rad) able to distinguish very small bands.

3.2.4 Hybridization, Scanning and Analysis

For each gene chip (mouse genome 430 2.0m array, Affymetrix) 20μg of fragmentated biotin-labelled cRNA was used.

The hybridization, scanning and further analysis was performed by the Scientific Support Services, Wolfson Institute for Biomedical Research, UCL, UK

The gene abbreviation obtained was identified further using the online software, SymAtlas version 1.2.4 from © Genomics Institute of the Novartis Research Foundation (http://symatlas.gnf.org/SymAtlas/about.jsp) which provides a description of the function of the gene, tissue distribution and links to pubmed (NCBI) for any reported scientific involvement of the gene.

3.3 QUANTITATIVE (REALTIME) PCR

Primers were designed using Netprimer (PREMIER Biosoft International) and PearlPrimer (SourceForge.net) and used with iQ™ SYBR® Green Supermix (Biorad 170-8882. All reactions were prepared on ice and run using a Biorad iCycler PCR machine. iQ™ SYBR® Green Supermix (Biorad 170-8882

Primers:

$Na_v1.8$

Scn10a s	5'-TGCAGATGCCTACACTGAGAAA-3'
Scn10a as	5'-AGTGAGCTAAGGATCGCAGAAA-3'

β-Actin

qActb s	5'-TTCTTTGCAGCTCCTTCGTT-3'
qActb as	5'-CGATGGAGGGGAATACAGC-3'

$TrpC_6$

$TRPC_6$ s	5'-ATTGATAAGGAGAGCGATGAGG-3'
$TRPC_6$ as	5'-AGGTCTTCTGTGTTCTGTGATTTC-3'

Edg7

Edg7 s	5'-TCCCATGAAGCTAATGAAGACAG-3'
Edg7 as	5'-TTCATGACGGAGTTGAGCAG-3'

$TrpV_1$

$TrtpV_1$ s	5'-CATCATCAACGAGGACCCAG-3'
$TtrpV_1$ as	5'-AACCAGGGCAAAGTTCTTCC-3'

$TrpM_8$

$TrpM_8$ s	5'-CGTATCATTTAGGAAGAAACCCA-3'
$TrpM_8$ as	5'-GAGGAAGGCGATGTAGAAGAC-3'

Programme:

Step 1	3 min	95°C
Step 2	30 sec	95°C
Step 3	30 sec	60°C
Step 4	For ever	4°C

Step 2-3 repeated 40 times.

Programme (melt curve)

Step 1	1 min	95°C
Step 2	1 min	55°C
Step 3	10 sec	55°C (increasing by 0.5°C per cycle)
Step 4	For ever	4°C

Step 3 repeated 80 times.

3.4 IMMUNOHISTOCHEMISTRY

3.4.1 Perfusion-protocol

After deep anaesthesia with Euthatal (150 mg/kg, i.p., Rhone Merieux), animals were transcardially perfused with 10 ml heparinized saline (0.9% w/v NaCl) followed by 25 ml of freshly prepared 4% w/v paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. The DRG were post-fixed in the same fixative solution for 2 hours at 4°C and then cryoprotected overnight in 30% w/v sucrose containing 0.02% sodium azide in 0.1 M PB at 4 °C. These perfused DRG were embedded in OCT embedding compound (BDH) on dry ice and stored at -80 C.

For dissection of the spinal cord, the vertebrae were cut to access the intact spinal cord. The axons entering the spinal cord were further cut before the spinal cord was removed and treated in the same way as DRG described above. For the freezing in OCT the spinal cord was placed to make cross sections possible.

When using fresh tissue, the dissected DRG were frozen down in blocks of OCT and kept at -80°C.

3.4.2 Sectioning

DRG was sectioned using a cryostat (Bright, UK). The 8-11 µm sections were mounted on electrostatically charged slides (Superfrost Plus, BDH) and left to dry for around 30 min at RT. For non-fixed sections, the slides were placed in ice-cold 4% PFA for 5-10 min.

Spinal cord was sectioned at 20µm and placed on Superfrost slides ad described above.

3.4.3 Staining

Slides were washed three times for 5 min in 1xPBS and placed in an immunohistochemistry perspex tray. For each slide, 200 µl blocking solution (PBS/10% Goat serum (Gibco BRL)) was added and left at RT for 1-2 hours. The blocking solution was replaced by 200 µl of the primary antibody diluted in PBS containing 0.1% triton-X100 (PBST) and left at RT over night. The slides were then washed 3-6 times in 1X PBS.

To each slide 200µl of secondary antibody diluted in PBST was added and left in the dark room at RT for 1-3 hours. A further three washes in 1X PBS were carried out before a drop of Aqueous Mounting solution (Sigma) or Citiflour (Citiflour Ltd, UK) was used to mount the coverslip (BDH).

The slides were analysed using a Leica epifluorescent microscope and Openlab software. The pictures were opened and merged using GIMP (Version 2.2.12.) software.

Primary antibodies

Antibody	Tissue	Dilution
IB4 – Isolectin B4 biotin labelled (Sigma, UK, L2140)	Perfused	1/200
CGRP (Sigma, UK)	Perfused	1:5000
Peripherin, IgG mouse monoclonal anti peripherin (Sigma, UK, P-5117)	No perfusion	1:800
Neurofilament, IgG Rabbit anti neurofilament 200 (Sigma, UK, N4142)	No perfusion	1:1000
N52, Sigma	Perfused	1:1000
$Na_v1.8$, SNS11 (Okuse et al., 1997)	No perfusion	1:1000
Substance P, IgG Rabbit anti-SP	Perfused	1:500

Secondary antibodies

Streptavidin conjugated Alexa488 (Sigma, UK)	1:1000
Alexa594, monoclonal goat anti rabbit IgG (Invitrogen, UK, 11037)	1:1000
Alexa488, Alexa flour 488, goat anti mouse IgG (Invitrogen, UK, A11017)	1:1000

3.4.4 Counting

Intact L4, L5 or L6 DRG from non-perfused WT and KO were sectioned at 11µm thickness as described above. They were labelled with anti-peripherin and neurofilament primary

antibodies. The secondary antibodies, goat anti mouse Alexa 488 and goat anti rabbit Alexa 594 were used for detection of primary antibody binding and after mounting the number of stained cells was quantified for each 8 sections. The result was expressed as the mean number of positive cells for each of the lumbar levels.

3.5 BEHAVIOUR PROTOCOLS

3.5.1 Animal Breeding Strategy

Mice were housed and bred at the Biological Services Unit animal facility at University College London under conditions complying with the Animals (Scientific Procedures) Act 1992. Homozygous DTA-mice (DTA -/-) were crossed with heterozygous $Na_v1.8$Cre (Cre +/-) mice to generate a 50/50% mix of DTA-CRE (DTA +/-, Cre +/-) mice and DTA-control (control littermates) (DTA +/-, Cre +/+). Throughout this thesis the DTA-CRE and DTA-control will refer to each genotype.

3.5.2 Injections of drugs

Compounds injected were diluted to the desired concentration using sterile saline (Sigma, UK). A Hamilton syringe (1710 TLL, 100µl) with disposable needles (Gauge 32, BD Microlance) was used for all injections of formalin, CFA, Carrageenan, Capsaicin and NGF. The PhlTx1 was injected using XX . Saline was used for all dilutions.

3.5.3 Rotarod Protocol

Before any nociceptive testing was performed both DTA-CRE and DTA-control mice were tested to ensure their motor function and co-ordination were intact using the Rotarod test (Ugo Basile).

The rod was accelerated up to 20rpm before placing the mouse on the rod. After 60 sec. at 20 rpm the rod was accelerated further until reaching a maximum of 36 rpm within a further 90 sec. The total length of time each animal spent on the rod was measured, having a cut off time of 300 sec.

The result was expressed as the mean time each group of mice spent on the rod ± the standard error of the mean (S.E.M.).

3.5.4 Weighing Protocol

As a further pre-nociceptive testing the weight of each mouse was monitored to identify any difference in weight gain between DTA-CRE mice and DTA-control mice.

Each mouse was placed in a bowl on a scale and the weight monitored. The result was expressed as the mean weights for each group of mice ± the standard error of the mean (S.E.M.).

3.5.5 von Frey Protocol

The mice were tested in groups of 12 and acclimatised in elevated perspex chambers with a metal mesh floor for 60-90 min or until any exploratory behaviour had stopped.

The von Frey filaments (Scientific Marketing Associates, UK) were applied using the Chaplan "up and down paradigm" (Chaplan *et al.*, 1994). Measured response to a hair filament would be the withdrawal of the paw from the applied hair. In general, the lack of response to the application of one hair-filament would lead to the application of a thicker hair. Likewise, a response would lead to the next application being of a thinner size. The experiment would stop when 6 responses had been monitored around the 50% threshold. All experiments were initiated using filament 7 (0.6g).
The 50% threshold were calculated using DIXON method and the result expressed as the mean value± the standard mean error (S.E.M.) (Dixon, 1980).

3.5.6 Randall Selitto Protocol

This test was carried out working with one mouse at a time. The mouse was placed in a perspex restrainer and left to settle for 5-8 min. The tail was gently placed under the probe of the Randall Selitto instrument (Ugobasile, Italy) before an increasing weight was applied to the tail. The weight for which the mouse would withdraw its tail was monitored. The test

was repeated three times on three different locations of the tail, at ½, ¼ and ¾ down the tail starting from the root with around one minute between each measure. The cut-off for all Randall Selitto testing was 500 g.

The result was expressed as the mean withdrawal latency in grammes for each group ± the standard error of the mean (S.E.M.).

3.5.7 Hargreaves' protocol

The mice were tested in groups of 12 and acclimatised in perspex chambers with a glass floor for 30-60 min until no exploratory behaviour was observed.

The Hargreaves heat source (IITC Life Science, Woodland Hills, CA, USA) was placed with the guide light pointing towards the plantar surface of the left hind paw and the thermal beam was turned on. A paw withdrawal would stop the test and the time recorded. The cut-off time for all testing using the Hargreaves instrument was determined at 20 sec. The test was repeated three times for each animal allowing at least 5 min. between each test.

The result was expressed as the mean withdrawal latency time for each group ± the standard error of the mean (S.E.M.).

3.5.8 Hot plate Protocol

Each of the mice from one cage of mixed genotype was acclimatised separately on the Hot Plate instrument (Ugo Basile, Italy), but without any power supply, for 15 min. The Hot Plate was heated until 50°C or 55°C and each mouse placed individually on the plate until lifting of the hind paws was observed and the latency thereof monitored.

The result was expressed as the mean withdrawal latency for each group ± the standard error of the mean (S.E.M.).

3.5.9 Noxious cold stimulation

3.5.9.1 Cold plate protocol

An incremental hot cold plate analgesia meter (IITC Life Science, Woodland Hills, CA, USA) was used to assess noxious cold sensitivity on the plantar surface of the paw as previously described (Kwan *et al.*, 2006;Zimmermann *et al.*, 2007). Mice were placed on a cold surface maintained at 0 ± 0.5°C, and pain was assessed by counting the number of hind paw lifts or jumping during a 5 min period. The mean of the nociceptive responses was determined in two separate trials taken at 60 min intervals to obviate thermal sensitization and/or behavioural interferences.

Results were expressed as the mean nocifensive responses for each group ± the standard error of the mean (S.E.M.). The results for the two groups were compared using an unpaired 2-tailed *t*-test

3.5.9.2 Acetone protocol

Mice were placed in the von Frey set-up and acclimatised for 1-2 hours. A drop of acetone (Sigma, UK) was applied using a 33 gauge needle inverted and connected to the syringe holding the acetone by a thin tube. The drop was applied to the plantar surface through the mesh and licking and biting behaviours were measured for 1 minute.

Results were expressed as the mean responses for each group ± the standard error of the mean (S.E.M.). The results for the two groups were compared using an unpaired 2-tailed *t*-test

3.5.10 Inflammatory protocols

Each mouse was used for only one inflammatory experiment and was culled afterwards according to the Animals (Scientific Procedures) Act 1992.

3.5.10.1 Formalin protocol

Before the injection of Formalin, a tissue was used to restrain the mouse. After the mouse had been gently restrained the left paw could be isolated and injected. 20 µl of 5% formalin (37% formaldehyde (BDH) in 0.9% saline (Sigma)) was injected subcutaneously into the

plantar surface of the left hind paw using a Hamilton micro syringe (1710 TLL 50µl). The mouse was observed in a perspex cage for the following 60 min monitoring pain behaviour, licking and biting of the injected paw.

The results were expressed in two different ways. First, the results were expressed as the mean time spent licking/biting in a 0-10 min interval and a 10-60 min interval. For each group ± S.E.M. The results for the two groups were compared using an unpaired 2-tailed t-test.
Secondly, the results from each 5 min. interval were expressed as the mean time spent licking/biting for each group ± S.E.M. and visualised in a graph showing the development in pain behaviour over the 60 min. Each time point was compared using an unpaired 2-tailed t-test. The data was also compared using a two-way repeated analysis of variance.

3.5.10.2 Carrageenan protocol

Baseline recordings of responses to thermal stimulation using the Hargreaves instrument were obtained before injecting each mouse with 20 µl of 2% Carrageenan in Saline (Both from Sigma, UK) into the plantar surface of one of the hind paws. Thermal thresholds were subsequently recorded using the Hargreave's instrument. The injected paw was measured after 30 minutes and every hour thereafter up to the 6^{th} hour.
For each time point of the experiment the data would be analysed as described for Hargreave's and compared with the baseline data using unpaired student t-test.

3.5.10.3 NGF protocol

Baseline recordings of responses to thermal stimulation using the Hargreave's instrument were obtained before 50ng of human recombinant NGF was injected into the plantar surface of the hind paw. Using the Hargreave's test, thermal responses were measured after 20, 40 and 60 minutes and for each hour until the 7^{th} hour.
For each time point of the experiment the data would be analysed as described for Hargreave's and compared with the baseline data using unpaired student t-test.

3.5.10.4 CFA protocol

Baseline responses for mechanical and thermal stimulation were obtained according to the protocols for von Frey and Hargreave's methods.

Mice were restrained and injected as described in the formalin protocol. 20 µl of Freund's Complete Adjuvant (Sigma F-5881, UK) was injected into the plantar surface of one of the hind paws. On day 1, 2, 5 and 8 mechanical (von Frey) and thermal (Hargreave's instrument) responses were obtained.

For each day of experiments the data would be analysed as described for von Frey and Hargreave's and compared with the baseline data.

3.5.10.5 Capsaicin protocol

Capsaicin was resuspended in DMSO and diluted in saline to obtain a working concentration of 0.05 µg/µl. The mice were acclimatised in small enclosed spaces (the Hargreave's set-up) for 1 hour before injecting one mouse at the time with 10 µl (0.5µg) capsaicin. The mouse was immediately observed for up to 5 minutes measuring licking and biting of the injected paw.

Results were expressed as the mean responses for each group ± the standard error of the mean (S.E.M.). The results for the two groups were compared using an unpaired 2-tailed *t*-test

3.5.11 Surgical procedures

The surgical procedures were performed in a designated theatre using a stereo zoom microscope (Olympus SZ40, on base SZ-STU1). After the procedure the animals were left to recover in separate cages and monitored for any adverse reactions before being moved back to their original room.

3.5.11.1 Chung protocol

Baseline responses for mechanical and thermal stimulation were obtained according to the protocols for von Frey and Hargreave's methods.

Animals were anaesthetised using Halothane (REF). A midline incision was made in the skin of the back at the L_2-S_2 levels and a further incision through the left paraspinal muscles was made to access the transverse processes at the L_4-S_1 levels. The L_5 transverse process was removed using a blunt fine forceps and the left L_5 spinal nerve was cut. The muscle tissue was closed with 4-0 mersilk sutures (Ethicon, UK) and clips were used to close the skin (Michel suture clip, 7.5 mm, FST, UK).

Responses to mechanical stimulation were assessed according to the protocol for von Frey filaments at 3, 5, 7, 10, 13, 17, 24 and 31 days after surgery. Responses to thermal stimulation were tested on day 4, 11, 18 and 25 according to the protocol for Hargreave's.

Results from the responses to thermal stimulation were expressed as the relative change in the latency at each time point after surgery (test/baseline) for each group ± S.E.M. The results for the two groups were compared using a two-way repeated measures analysis of variance test (ANOVA).

Results from the responses to mechanical stimulation were expressed as the relative change in the 50% threshold at each time point after surgery (test/baseline) for each group ± S.E.M. The results for the two groups were compared using a two-way repeated measures analysis of variance test (ANOVA).

3.5.11.2 Seltzer protocol

Baseline recordings were obtained as described in the Chung protocol.

Animals were anaesthetised using Halothane. An incision was made in the skin of the upper left leg and blunt scissors were used to part the muscle layers to access the sciatic nerve. A tight ligation of between ½-⅓ of the nerve was made using 6-0 mersilk suture (Ethicon, UK). The skin was closed using 4-0 mersilk sutures (Ethicon, UK).

After at least 3 days of recovery the mice were tested and analysed as described for the Chung protocol.

3.5.12 Spontaneous pain

Each mouse was restrained as described in the protocol for formalin. 20 µl of Freund's Complete Adjuvant (Sigma F-5881, UK) was injected subcutaneously into the paw. For the second injection, the fur to the side of the knee joint was shaved off and another 20µl of CFA was injected subcutaneously at the side of the right knee. On day 1, 2, and 4 each mouse was acclimatised for 10-30 minutes in a perspex cage and observed for 2 times 5 minutes and the number of spontaneous paw lifts monitored.

The result was expressed as the mean number of behaviours for each group ± the standard error of the mean (S.E.M.) for each day.

3.6 CALCIUM IMAGING

3.6.1 DRG culture

DRG from DTA-control and DTA-CRE mice were dissected and incubated in an enzyme digestion mixture (PBS, Collagenase, Dispase, Glucose) for 30 min at 37°C. The DRG were washed twice in culture medium and titurated using a Gilsson P1000 pipette to separate the cells before being passed through a 100 µm pore cell-strainer (Falcon, UK). The cells were pelleted by spinning the suspension for 5 minutes at 1K rpm. The cells were resuspended in 300-600 µL medium and drops of 30 µl cell suspension were cultured on poly-L-lysine and laminin coated cover slips in 35 mm petri dishes. After 60 minutes the cover slips were flooded in Dubecco's modified Eagle Medium (DMEM) containing 10% heat-inactivated foetal bovine serum (FBS/Gibco) and 10.000 IU/ml penicillin/streptomycin (Gibco). The cultures were used two days after preparation.

3.6.2 Imaging

Intracellular free calcium was measured using dual excitation fluorescence of the calcium sensitive probe Fura-2. Fura-2 was loaded into the cell using the acetoxymethyl-ester form of the probe (Fura-AM, 5uM, 30min, 37°C). Images of cells were recorded using a 12-bit grey-scale camera (Hammamatsu 8480) controlled by OpenLab acquisition software. Images were obtained at excitation wavelengths of 360 and 380 nm and emitted light captured at 520 nm. Exposure times were normally within the range 250 to 2500 msec. Post acquisition analysis was undertaken also using OpenLab where 360 and 380 nM images were ratioed after background subtraction. In single-cell high-resolution experiments the 360/380 ratio images were subjected to a calibration procedure using the equation $[Ca^{2+}]_I = Kd^* (R-R_{min}) / (R_{max}-R)$. the parameters Kd* (1055), Rmin (.33) and Rmax (1.99), determined in separate experiments (Winks et al, 2005). More often the 360/380 ratio data was utilised; this was estimated from within defined areas of the cytoplasm (>25 um^2).

Prior to any imaging, the cultures were loaded with 1 μL of the membrane permeable acetoxylmethyl-ester form of the Ca^{2+} sensitive dye Fura-2 (2 μM in 0.1% pluronic F-1278, Molecular Probes, Eugene) and incubated for 60 min at RT. Following incubation, the cells were rinsed in isotonic external solution, ECF, (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, pH 7.4 with NaOH) and allowed 10–20 min to de-esterify the dye. The Fura-2-loaded neurons were exposed to a constant flow of ECF. Capsaicin, 1μM (Roche, UK) was applied for 30 seconds before being washed continuously using the ECF. When activating with Menthol, 100μM (Sigma, UK) was applied for 1 min before washed continuously in ECF.

Using a 10X objective, groups of neurons were observed on an inverted microscope (Zeiss Axiovert 200). Neurons were exposed to epifluorescence illumination using a Polychrome IV system (T.I.L.L. Photonics GmbH, Gräfelfing, Germany) with a 150 W xenon lamp with light of alternating excitation wavelengths (340 and 380 nm). Emission fluorescence (F) was led to an IMAGO CCD camera through a dichroic filter and a 440 nm longpass filter. Fluorescent image intensities were expressed as the ratio F340/F380 to allow quantitative estimates of changes in intracellular calcium levels. F340 and F380 represent the fluorescence intensity elicited by 340 nm emission light (for Fura—Ca^{2+}) and 380 nm

emission light (for Fura without Ca^{2+}) respectively. All experiments were performed at room temperature (23–25 °C).

All data were normalised and expressed as the mean number of Capsaicin (XX) responding cells for each group ± the standard error of the mean (S.E.M.).

3.7 ELECTROPHYSIOLOGICAL INVESTIGATIONS

3.7.1 Whole cell patch clamping recordings

Voltage-clamp recordings from cultured DRG neurons were carried out at a holding potential of -60 mV using an Axopatch 200B amplifier (Axon Instruments, Inc.). Pipettes were pulled from borosilicate glass capillaries with a P-97 puller (Sutter Instrument Co.) and exhibited resistances of 1-3 MΩ. Currents were digitized with a Digidata 1322A data acquisition system (Axon Instruments, Inc.).

Data was recorded and stored using Clampex 8.1 (Axon Instruments, Inc.). Currents were low pass-filtered at 2 kHz and sampled at 11 kHz. Capacity transients were cancelled, and series resistance was compensated by 80% minimum. Voltages were not corrected for liquid junction potentials. Recordings were performed at room temperature. Off-line analysis and statistics were performed using Clampfit 9.0 (Axon Instruments, Inc.), SigmaPlot 8 (Systat Software Inc.) and QuickCalcs (GraphPad software). Values were expressed as means ± S.E. The standard pipette solution contained 140 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES (pH 7.3). The standard external solution contained 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4).

3.7.1.1 Mechanical stimulation of DRG

The probe used to stimulate the neurons mechanically was a heat-polished glass pipette (tip diameter ~5-6 μm) positioned at an angle of ~ 60° to the surface of the culture dish. The probe was connected to a computer-controlled piezo-electric crystal drive (Burleigh) and positioned such that a 10 μm movement did not visibly contact the cell but an 11 μm stimulus produced an observable membrane deflection. A 1 μm stimulus was attributed to a probe movement of 11 μm stimulation, a 2 μm stimulus to a 12 μm movement and so on. The probe moved at a speed of 0.5 μm/ms and the stimulus duration was 200 ms. The

correlation between action potential properties and DRG neuronal phenotype that exists in adult animals was used to distinguish between mechanoreceptive (low threshold) neurons and nociceptive (high threshold) neurons. IB4 staining was used to distinguish between peptidergic and non-peptidergic $IB4^+$ small neurons.

3.7.2 Mouse spinal cord electrophysiology *in vivo*.

Intact anaesthetised male and female mice (8 – 10 weeks) were used in all experiments, in accordance with the requirements of the United Kingdom Animals (Scientific Procedures) Act (1966). After briefly inducing anaesthesia with 3 % halothane (66 % N_2O and 33 % O_2), mice were injected with urethane (240 mg/kg). Animals were placed in a stereotaxic frame and a laminectomy was performed to expose the L3 – L6 spinal segments. Extracellular recordings were made from single wide dynamic range (WDR) neurones using parylene-coated tungsten electrodes (A-M Systems, USA). Neurones were visualized on an oscilloscope, and were isolated and discriminated by spike amplitude and waveform. All neurones had receptive fields over the hind paw. Depth of the neurone was measured from the surface of the spinal cord.

Electrical stimulation was administered by inserting two needles into the receptive field. A train of 16 stimuli was given (2 ms pulse duration, 0.5 Hz at three-times the C-fibre threshold). The neuronal responses evoked were superimposed and separated on the basis of fibre conduction velocity and latency into total A-fibre-evoked (0 – 50 ms), and C-fibre-evoked (50 – 250 ms) action potentials. Neuronal responses occurring after the C-fibre latency band (250 – 800 ms) were classed as post-discharge. The 'input' (non-potentiated response) and the 'wind-up' (potentiated response, shown by increased neuronal excitability to repeated stimulation) were calculated as follows: Input = (action potentials (50 – 800 ms) evoked by first pulse at 3 times C-fibre threshold) – total number of pulses (16). Wind-up = (total action potentials (90 – 800 ms) after 16 train stimulus at 3 times C-fibre threshold) – Input.

A wide range of natural stimuli including brush; von Frey filaments (Scientific Marketing Associates, Barnet, UK) in ascending order (1, 6, 8, 15, 26 and 60 g) and heat (35, 40, 45 and 50°C water jet), were applied to the receptive field for 10 seconds per stimulus. Data was captured and analysed by a CED 1401 interface (Cambridge Electronic Design,

Cambridge, UK) coupled to a Pentium computer with Spike 2 software (post-stimulus time histogram (PSTH) and rate functions).
Data was analysed from a total of 17 wide dynamic range (WDR) neurones in DTA-CRE mice (n=8) and DTA-control littermate controls (n=9). All neurons selected responded to innocuous and noxious stimuli.
Statistical analysis was conducted using unpaired t-tests to compare evoked responses of DTA-CRE and their DTA-control.

3.8 GENERAL REAGENTS

All general reagents were obtained from Sigma or BDH.
All tools for surgery and dissections etc. were obtained from Fine Science Tools, UK

The Diphtheria toxin fragment A floxed transgenic mouse line was a kind gift from Professor Andy Kopp, UCL Institute of Child Health.

The Tarantula spider toxin, PhlTx1 was a gift from Dr Pierre Escoubas, University of Nice – Sophia Antipolis, Institute of Molecular and Cellular Pharmacology, Valbonne, France.

The behavioural work on the transgenic PAPIN mouse line was performed at the University of Hong Kong, Department of Biochemistry, Faculty of Medicine Building, Hong Kong.

4 DELETION OF $NA_V1.8$-EXPRESSING NEURONS

4.1 INTRODUCTION – DIPTHERIA TOXIN

Historically, the toxicity of diphtheria has been exploited in scientific studies by direct micro injections into tissues to introduce nerve lesions. Harrison et al. used injections of diphtheria toxin (DT) into cats to study remyelination in the CNS (Harrison et al., 1972). Another example of the early use of Dipththeria toxin is described in the paper by Baker and Bostock using intrathecal injections of DT into rats to investigate Ectopic discharges after demyelination (Baker and Bostock, 1992).

This chapter describes how a transgenic mouse line is generated to target the expression of diphtheria toxin to the $Na_v1.8$ specific population of sensory neurons. The deletion in tissue is confirmed and characterised using immunohistochemistry and responses to different painful stimuli are assessed. Finally, the changes in the expression of genes in DRG are investigated using microarray on RNA isolated from DRG.

4.1.1 Diphtheria toxin mechanism of action

Diphtheria toxin is a two-fragment toxin consisting of an A-fragment and a B-fragment. The B-fragment binds to a specific cell surface receptor and enables the toxin to insert into the phospholipid bilayer. This facilitates transfer of the enzymatic A-fragment across the membrane. The A-fragment catalyses the transfer of the ADP-ribose moiety of Nicotinamide adenine dinucleotide (NAD) to Elongation factor-2 (EF-2), thus inhibiting protein synthesis (Collier, 2001). Sensitivity to Diphtheria toxin differs between species owing to the lack of cell surface receptors in some species. In rat and mouse in particular the sensitivity is very low, around 10,000 fold less than, for example; humans and monkeys (Eidels et al., 1983).

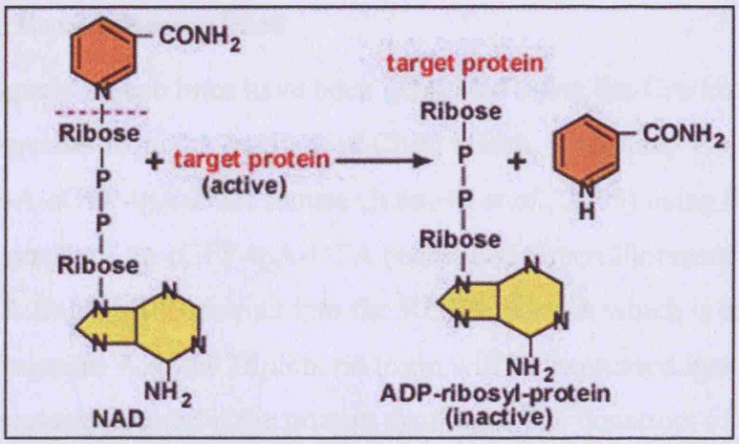

Figure 9. The mechanism for diphtheria toxin cell death. Diphtheria toxin catalyses the ADP-ribosylation of the elongation factor (EF-2) resulting in inactivation of EF-2.

Diphtheria toxin has been used in varying ways to investigate the deletion of specific cell populations. In recent years several mouse lines have been generated with the aim of expressing Diphtheria toxin fragment A (DTA) linked to a tissue-specific promoter. In some cases deletion occurred successfully, but examples of incomplete deletion giving rise to insignificant phenotypes have been reported (Breitman et al., 1987). It has been mooted that random integration was the cause due to the pronucleus microinjection, leading to the deletion of unrelated cells (Ivanova *et al.*, 2005).

4.1.2 The use of Diphtheria toxin in the mouse

For decades diphtheria toxin has provided a platform for research studies, in recent years more sophisticated techniques have been developed to target specific cells. The breeding of transgenic mice to target specific cells has been used extensively. One example is the study from Chen et al. which exploited the lack of sensitivity of DT in mice to study the olfactory sensory neurons after controlled ablation. By introducing the human diphtheria receptor under the control of the olfactory marker protein promoter, subsequent injections of diphtheria deleted the olfactory neurons (Chen et al., 2005). Another method by which specific cells can be targeted is the Cre/*lox*P system as described below.

4.1.3 The DTA floxed mouse lines

A number of transgenic mouse lines have been generated using the Cre/*lox*P system in recent years. Researchers from the Institute of Child health, University College London generated the ROSA-eGFP-tpA-DTA mouse (Ivanova *et al.*, 2005) using homologous recombination to introduce an eGFP-tpA-DTA (enhanced Green Flourescent Protein - Diphtheria Toxin A fragment) construct into the ROSA26 locus which is expressed ubiquitously. As fragment A of the Diphtheria toxin will be expressed inside the cell, only this fragment is necessary to inhibit the protein synthesis. The construct of the transgenic line is based on the insertion of two *loxP* sites flanking a sequence consisting of an eGFP and strong transcriptional termination sequence (tpA), followed by the DTA cassette – see fig.10. This will keep the expression of DTA silent until the flanked sequence has been excised by Cre. The ROSA-eGFP-tpA-DTA mouse was tested using two different Cre lines to confirm the successful use of Diphtheria toxin to delete cells in a tissue-specific way. At least two other transgenic mouse lines have been generated with the ability to express DTA in a tissue-specific manner using the Cre/*loxP* system (Collier, 2001;Ivanova *et al.*, 2005;Sato and Tanigawa, 2005)

4.2 DELETION OF $Na_v1.8$-EXPRESSING NEURONS

$Na_v1.8$ is expressed in the sensory neurons (nociceptors) and a Cre-expressing mouse line has been generated with Cre expression linked to the promoter of $Na_v1.8$. By crossing the $Na_v1.8$Cre mouse (section 2.3.2.3) with the floxed DTA mouse a tissue-specific deletion of the $Na_v1.8$ positive neurons was possible.

The effect of the deletion of the $Na_v1.8$-expressing sensory neurons was characterised functionally by using a host of nociceptive behaviour assays. Using immunohistochemistry, the efficiency of the deletion of $Na_v1.8$ expressing neurones on the DRG neurons were characterised. Finally, single cell electrophysiology was applied to investigate what effect the neuron ablation had centrally.

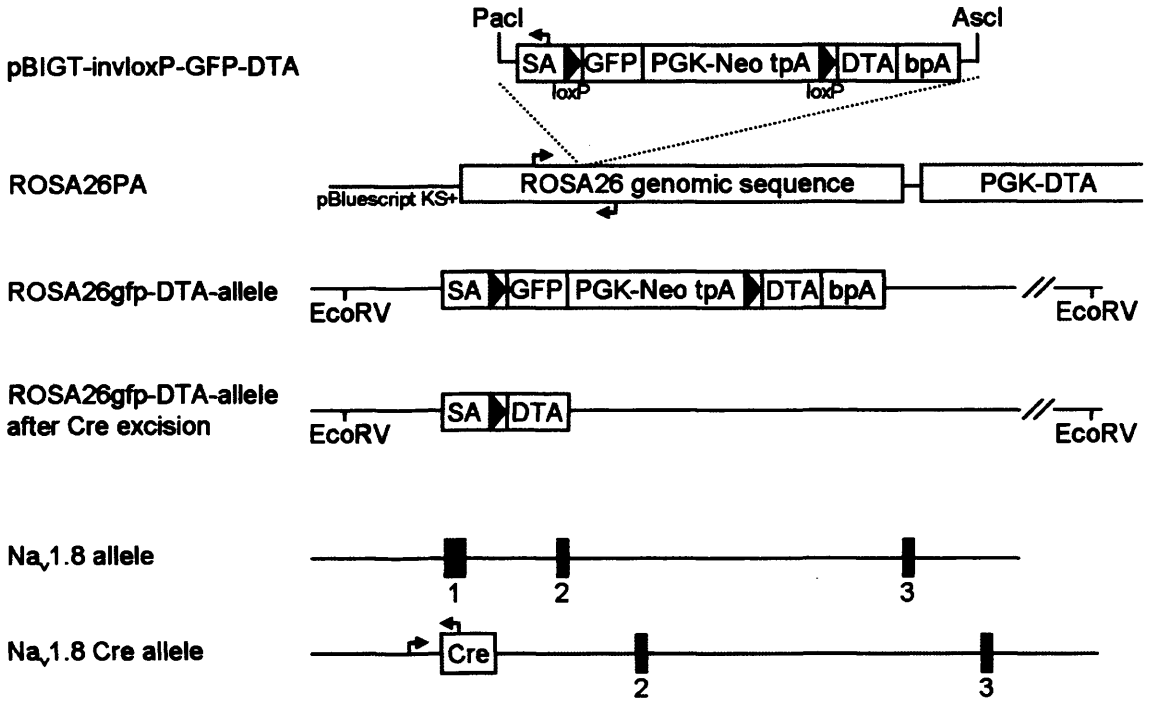

Figure 10. Overview of the DTA and Na$_v$1.8Cre mouse lines.
From top to bottom:
Insertion of the DTA-construct into the genomic ROSA sequence and the PGK-DTA cassette for negative selection.
Targeted ROSA-DTA sequence before deletion.
Targeted ROSA-DTA sequence after deletion.
Na$_v$1.8 genomic sequence – exon 1-3.
Targeted Na$_v$1.8Cre sequence.
(Ivanova *et al.*, 2005;Stirling *et al.*, 2005)

4.2.1 Results

4.2.2 Breeding and genotyping

Pairs of homozygous DTA floxed mice and heterozygous Na$_v$1.8Cre mice were bred. The genotype of the offspring was tested by PCR using primers for the floxed strong transcriptional termination sequence tpA (fig. 11) and for the Cre transgene (fig. 12) with frequencies following the Mendelian ratios.

The heterozygous floxed DTA mouse expressing Cre will throughout this chapter and discussion, be referred to as DTA-CRE. The heterozygous floxed DTA mouse not expressing Cre will be referred to as DTA-control.

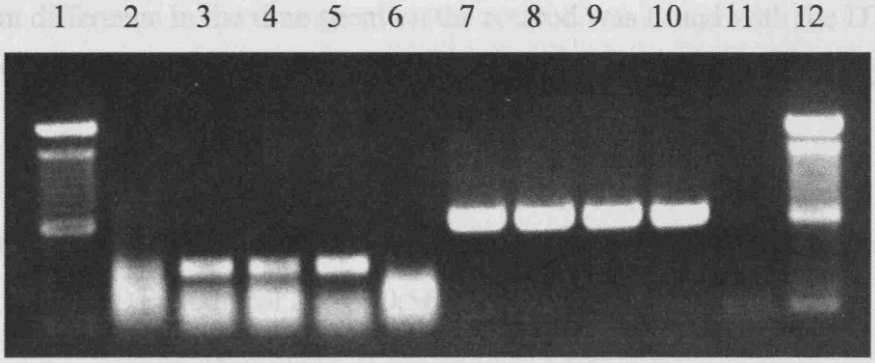

Figure 11. DTA floxed genotyping.
Marker (lane 1+12)
Floxed DTA band (250 bp, lane 2-5)
Floxed DTA band negative control (lane 6)
Wild type DTA band (600 bp, lane 7-10)
Wils type DTA negative control (lane 11)

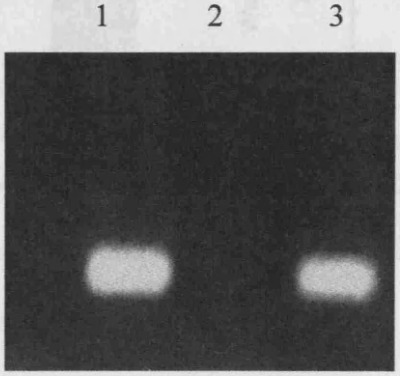

Figure 12. CRE genotyping.
Cre-positive band at 450 bp (lane 1+3)
When Cre is not expressed the PCR does not generate any band (lane 2)

4.2.2.1 Motor function and weight

Before any experiments were performed on the mice, differences in weight gain or motor function and coordination were investigated to ensure that the mice developed normally. None of the mice showed any overt differences in appearance or general behaviour. Their vital signs appeared unimpaired and were fertile generating litters of between 6 and 11 mice.

A group of 14 DTA-CRE mice and 9 DTA-control mice were tested on the rotarod to control the motor function.

No significant difference in the time spent on the rotarod was found with the DTA-CRE spending 171±14.55s and DTA-control 158±17.18s on the rod respectively (fig. 13 right).

9 DTA-CRE mice and 9 DTA-control mice of mixed gender were weighed (fig 13. left). No significant difference in the weight was found. The DTA-CRE weighed on average 23.74±1.57g and the DTA-control 22.05±0.56g.

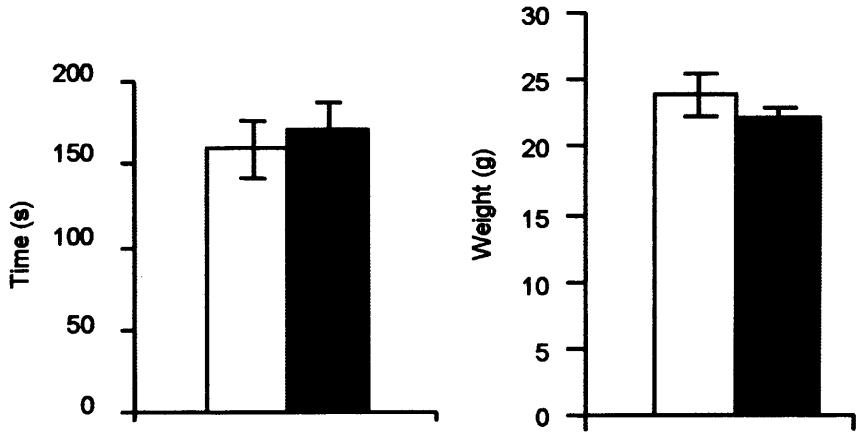

Figure 13. Motor function and weight for DTA mice
Rotarod (left) and weight (right).
■ DTA-CRE, □ DTA-control

4.2.3 Immunohistochemistry

Several experiments were performed to quantify and characterise the sensory neurons after deletion of the $Na_v1.8$-expressing neurones.
A quantification was done by counting the small sensory neurones labelled with anti-peripherin and the larger neurones labelled by anti-neurofilament. Antibody against $Na_v1.8$ was used to detect any remaining $Na_v1.8$ positive neurons to test for complete deletion.
A further characterisation of the remaining neurons was performed to identify the subpopulations of the small sensory neurones, the peptidergic and non-peptidergic, to observe what populations were present after deletion.

To check for unspecific binding of the secondary antibody, control of the secondary antibody without any primary antibody was carried out for all antibodies used.

4.2.3.1 88% reduction in peripherin positive cells in DTA-CRE mouse

The Peripherin/neurofilament staining of the small and large neurons respectively, from every 8 section from each Lumbar DRG L4, L5 and L6, were quantified from a DTA-CRE and a DTA-control mouse (fig. 14 and fig. 15). Two individual sets of sections were counted from each DRG. By counting every 90μm, a representative picture of the expression of the cells was obtained. Theoretically no cells would be counted more than once (large cells are <90μm) and even though a lot of small cells would not be counted, the same procedure were performed for both DTA-CRE and DTA-control mice.
The percentage loss of neurons in the DTA-CRE was calculated by dividing by the DTA-control and multiplying by 100.

	Neurofilament		Peripherin	
	WT	KO	WT	KO
L4-1	405	310	648	64
L4-2	368	381	595	96
Mean	387	345.5	622	80
KO/WT*100	**89.27%**		**12.89%**	

	Neurofilament		Peripherin	
	WT	KO	WT	KO
L5-1	364	332	708	105
L5-2	371	419	748	115
Mean	367.5	375.5	728	110
KO/WT*100	**97.87%**		**15.10%**	

	Neurofilament		Peripherin	
	WT	KO	WT	KO
L6-1	414	265	837	60
L6-2	367	321	907	62
Mean	414	293	872	61
KO/WT*100	**70.77**		**7.00**	

Pooled L4-L6		
Pooled L4-6	**86.77%**	**11.30%**

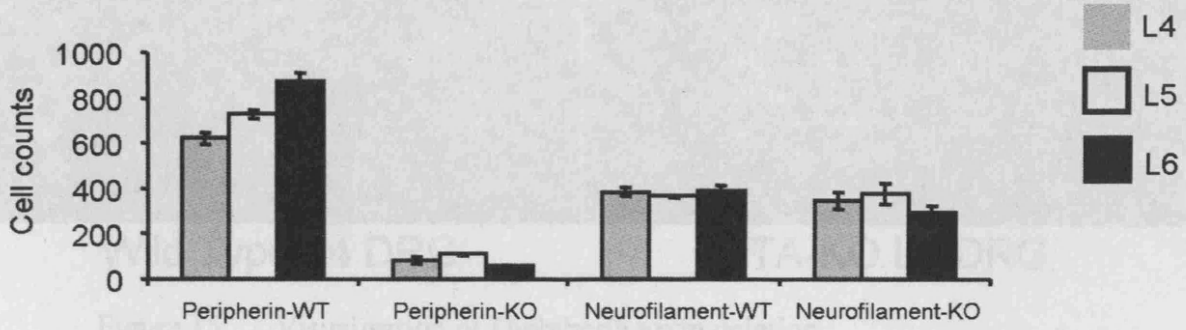

Figure 14. Cell comparison of DTA-control and DTA-CRE DRG

The counting between the DTA-CRE and DTA-control was tested using an unpaired t-test with the data pooled from the Peripherin staining and the neurofilament staining respectively.

In the DTA mice the number of neurofilament positive neurons was 86.77% of the DTA-control ($P<0.05$), whereas the majority of the peripherin positive neurons were deleted leaving only 11.30% ($P<0.001$) positive neurons compared with the DTA-control. This strongly indicates a very effective deletion of small sensory neurons in the DTA-CRE mouse by using the $Na_v1.8$cre crossed with the DTA mouse.

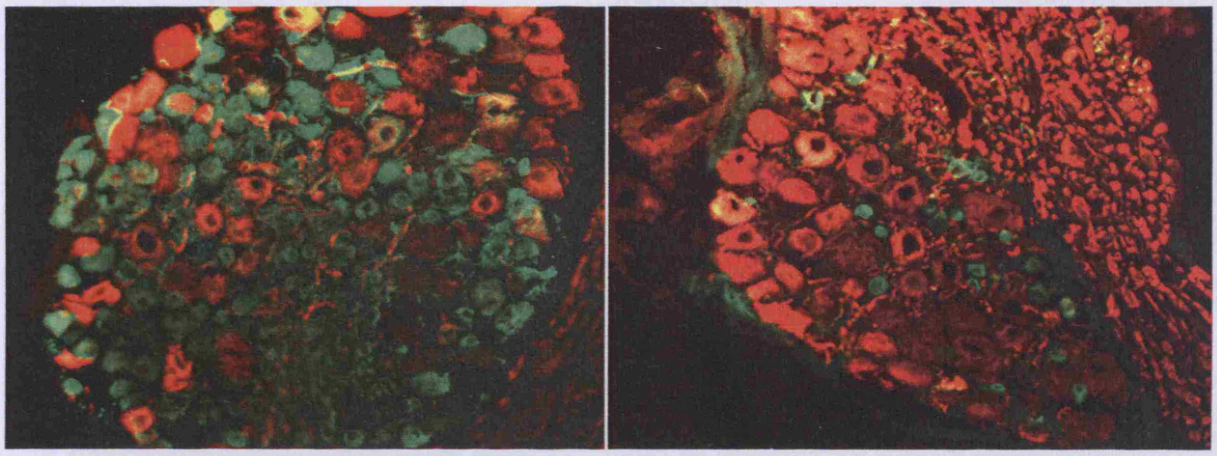

Figure 15. Visualisation of Diphtheria toxin deletion.
Neurofilament (red) and peripherin (green).

4.2.3.2 All $Na_v1.8$-expressing neurons ablated by Diphtheria toxin

A further control of the efficiency of the $Na_v1.8$Cre was to stain for the $Na_v1.8$ channel. The $Na_v1.8$ antibody SNS11 (Djouhri *et al.*, 2003) was used on lumbar DRG tissue sections from DTA-CRE and DTA-control mice.

In the figure (Fig.16) the SNS11 antibody is co-stained with peripherin. Peripherin will label all the small neurons and $Na_v1.8$ is expressed in around 85% of these (Djouhri *et al.*, 2003). In the DTA-control the $Na_v1.8$ is, as expected, co-localised with a large majority of the peripherin positive cells and a few large cells as well. Looking at the DTA-CRE sections, around 88% of the peripherin positive cells have been deleted. Of greater importance is the result that $Na_v1.8$ expression in the DTA-CRE mouse is almost completely ablated.

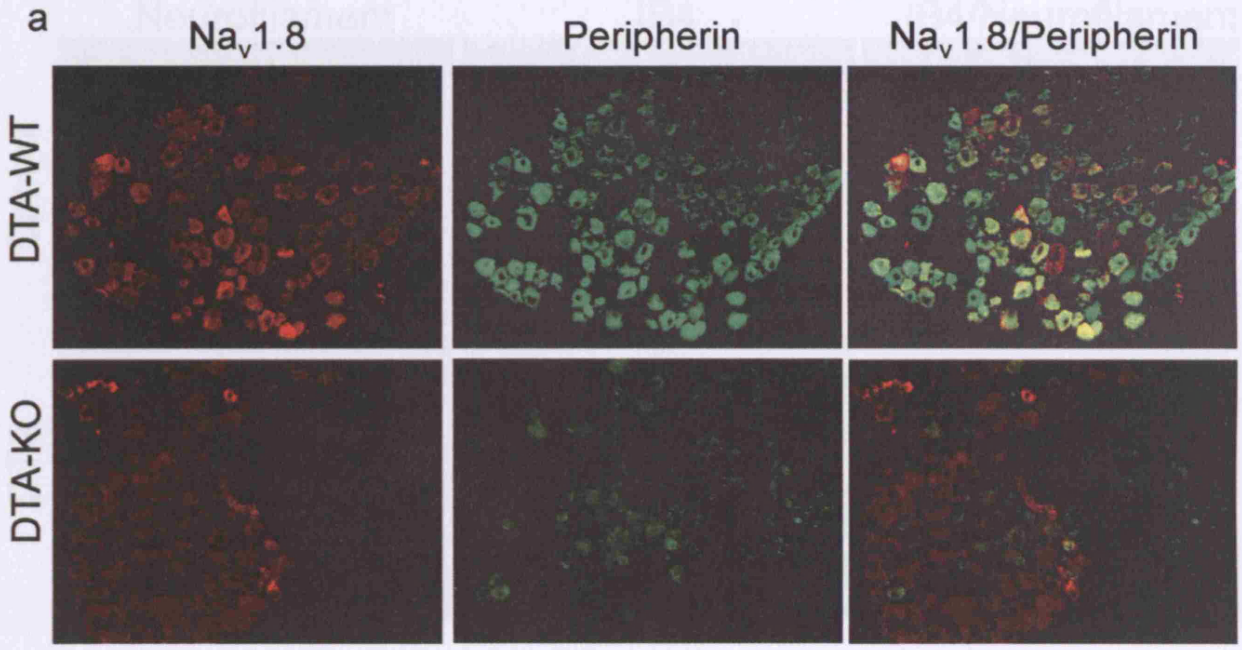

Figure 16. Na$_v$1.8 staining
DRG sections were stained with anti-Na$_v$1.8 (red) and anti-peripherin (green).

4.2.3.3 The majority of non-peptidergic neurons are deleted in the DTA-CRE

The small sensory neurons can be divided into two subpopulations. The peptidergic neurons express neuropeptides such as CGRP and Substance P and terminate centrally in lamina I and outer lamina II. The non-peptidergic population is characterised by binding of Isolectin B4 and does not synthesise any neuropeptides.

To characterise the remaining neurons further following deletion of the Na$_v$1.8-expressing neurons, sections were stained for the non-peptidergic (IB4) and peptidergic (CGRP) subtypes.

Staining with anti-Isolectin B4 (Fig. 17) revealed a complete absence of non-peptidergic neurons at DRG level suggesting that Na$_v$1.8 is expressed in all non-peptidergic neurons.

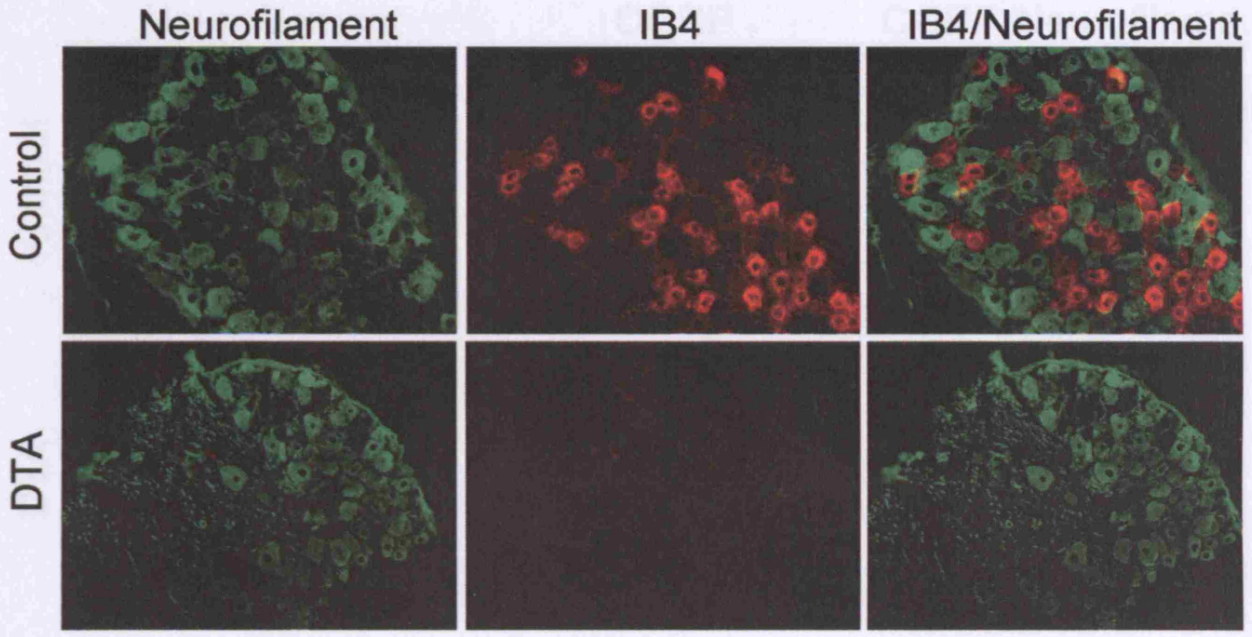

Figure 17. IB4 staining of DRG sections
DRG sections were stained with anti-neurofilament (green) and anti-IB4 (red).

4.2.3.4 Some peptidergic neurons remain in the DTA-CRE

To investigate if any peptidergic neurons were left in the DTA-CRE mouse, sections of DTA-CRE DRG were stained with an antibody against CGRP (Fig. 18).
The staining showed that in the DRG the majority of the CGRP positive neurons had been deleted in the DTA-CRE, but around 11% of remained present in the DTA-CRE when compared with the DTA-control mice. This suggests that at least some of the peptidergic neurons do not express $Na_v1.8$.

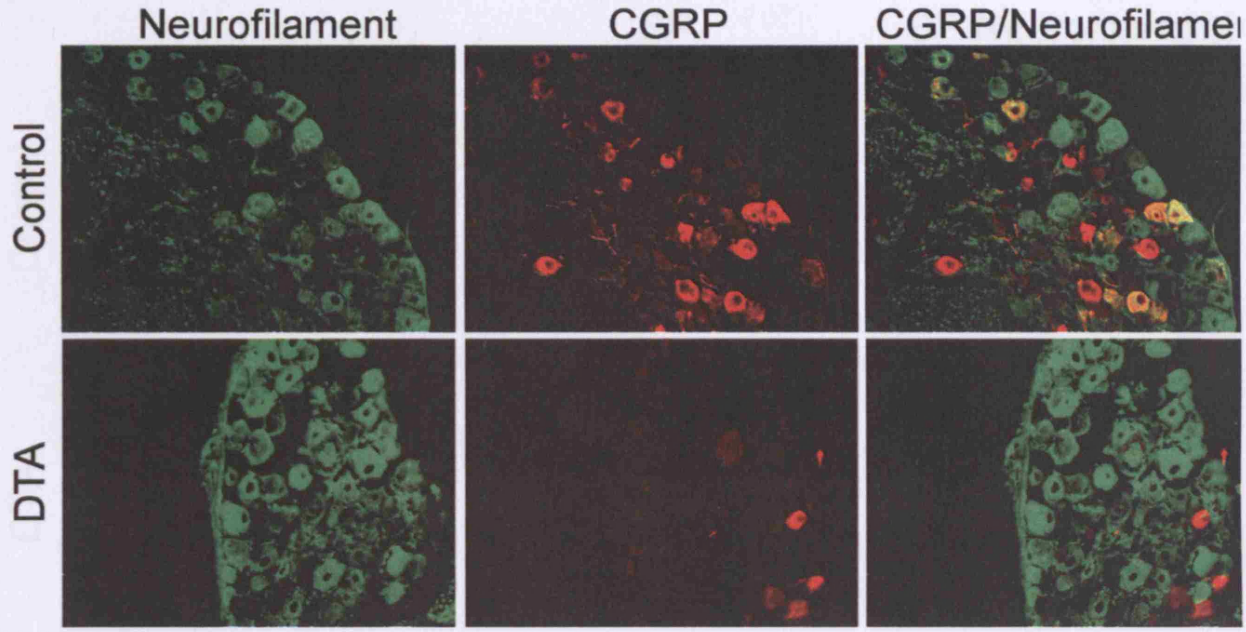

Figure 18. CGRP staining of DRG sections
DRG sections were stained with anti-neurofilament (green) and anti-CGRP (red).

4.2.3.5 Residual Substance P positive neurons in DTA-CRE

To investigate the characteristics of the remaining DRG neurons, in more detail, sections were stained with anti-substance P antibody (Fig. 19). In the DTA-control a large proportion of cells stained positive for the antibody. Interestingly, in the DTA-CRE only a very small number of cells, around 3%, indicated positive for substance P.

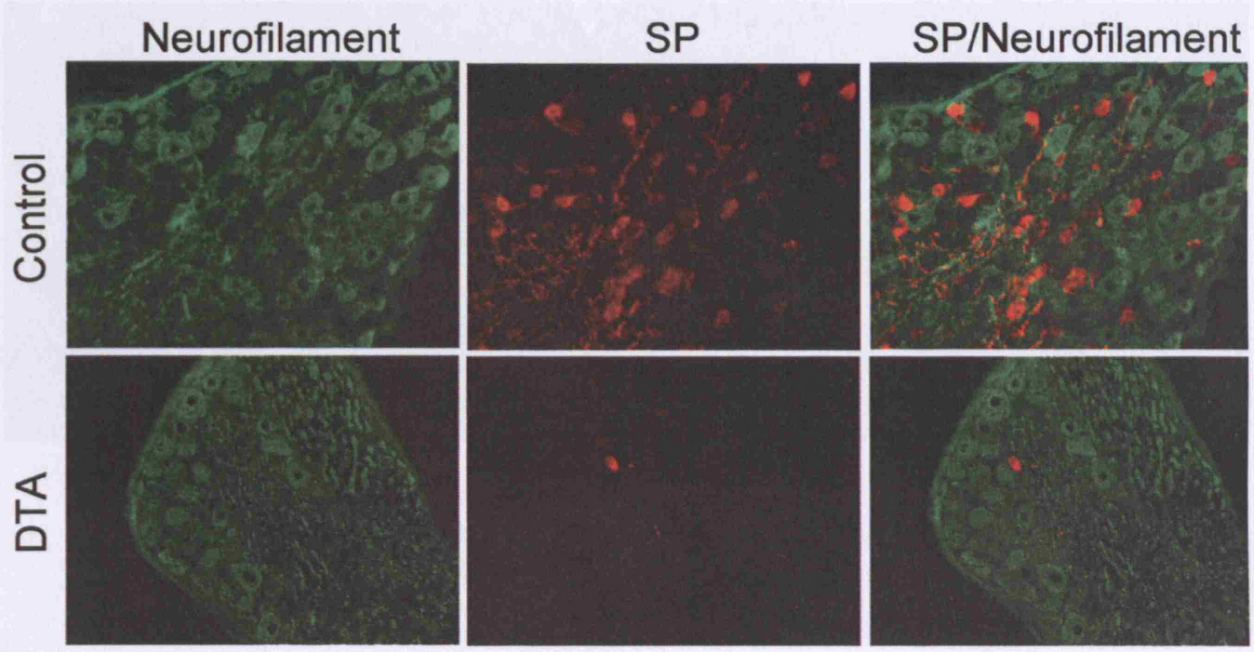

Figure 19. Substance P staining of DRG sections
DRG sections were stained with anti-neurofilament (green) and anti-SP (red).

4.2.3.6 Spinal cord staining

To investigate differences in dorsal horn termination of the small sensory neurons after ablation of the $Na_v1.8$-expressing neurons, spinal cord sections were stained with markers for the peptidergic (CGRP) and non-peptidergic (IB4) sensory neurons (Fig. 20).

In the DTA-control a very clear labelling of CGRP positive fibres was recorded. From the DTA-CRE sections a significant reduction in staining was observed. For the IB4 staining a complete absence of staining is seen in the DTA mouse when compared with a thin layer of staining in the DTA-control. This observation confirms the results from the DRG staining, that the non-peptidergic neurons have been deleted, but some peptidergic still remain in the DTA-CRE mouse.

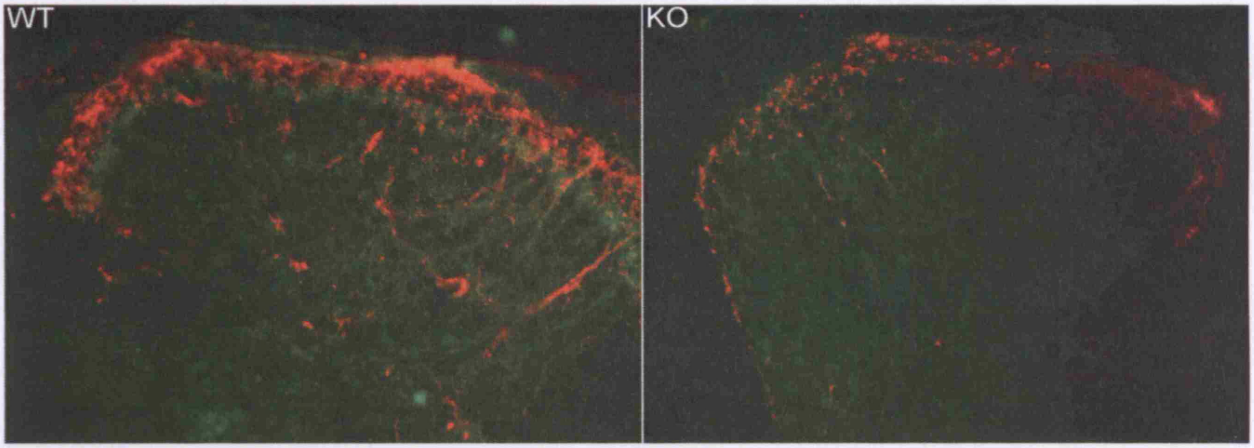

Figure 20. Dorsal horn staining
Cross-sections of spinal cord were stained with anti-CGRP (red) and anti-IB4 (green).
DTA-control (left) and DTA-CRE (right).

4.2.4 Calcium imaging

4.2.4.1 $TrpV_1$ positive neurons strongly reduced in DTA-CRE

DRG neurons from DTA-CRE and DTA-control mice were cultured and prepared for calcium imaging. By applying 1μM capsaicin for 30 seconds $TrpV_1$ positive cells were activated allowing influx of calcium. An infrared detectable fluorescent complex was formed between the calcium and the calcium dye FURA2 which made it possible to identify $TrpV_1$ positive cells (Fig. 21).

From the DTA-control 47.87±3.89% of the cells responded by an influx of calcium, confirming response to capsaicin, compared with only 9.28±2.33% in the DTA-CRE ($P<0.001$). The very low number of $TrpV_1$ positive cells in the DTA-CRE suggests a very close relationship between expression of $Na_v1.8$ and $TrpV_1$.

The result suggests that if $TrpV_1$ performs a role in acute thermal sensation and thermal hyperalgesia after induction of inflammation a behavioural phenotype is expected.

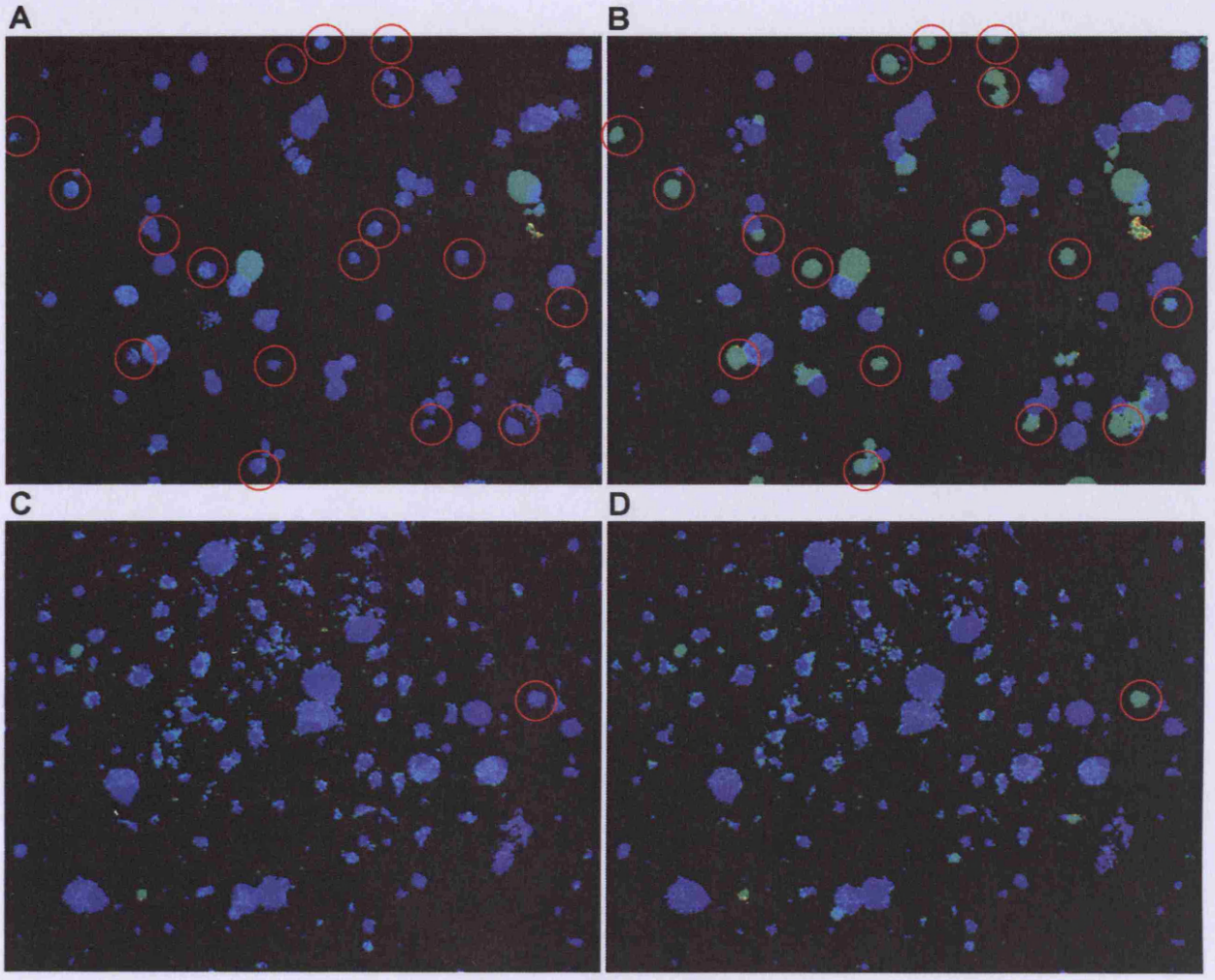

Figure 21. Calcium imaging – Capsaicin activation
Representative photo of the calcium imaging experiment.
DTA-control: before (a) and after (b) activation
DTA-CRE: before (a) and after (b) activation
.

4.2.4.2 Menthol positive neurons remains in DTA-Cre

For investigation of the TpmM8 responses in the DTA-CRE mouse, DRG cultures were prepared as above, but activated with 100µM Menthol for 1 min (Fig. 22).

Interestingly, a large proportion of the remaining small cells in the DTA-CRE mouse responded to menthol. Approximately 75% of the menthol positive cells seen in the DTA-control remained in the DTA-CRE mouse. This suggests that the majority of $TrpM_8$ is not co-localised with $Na_v1.8$.

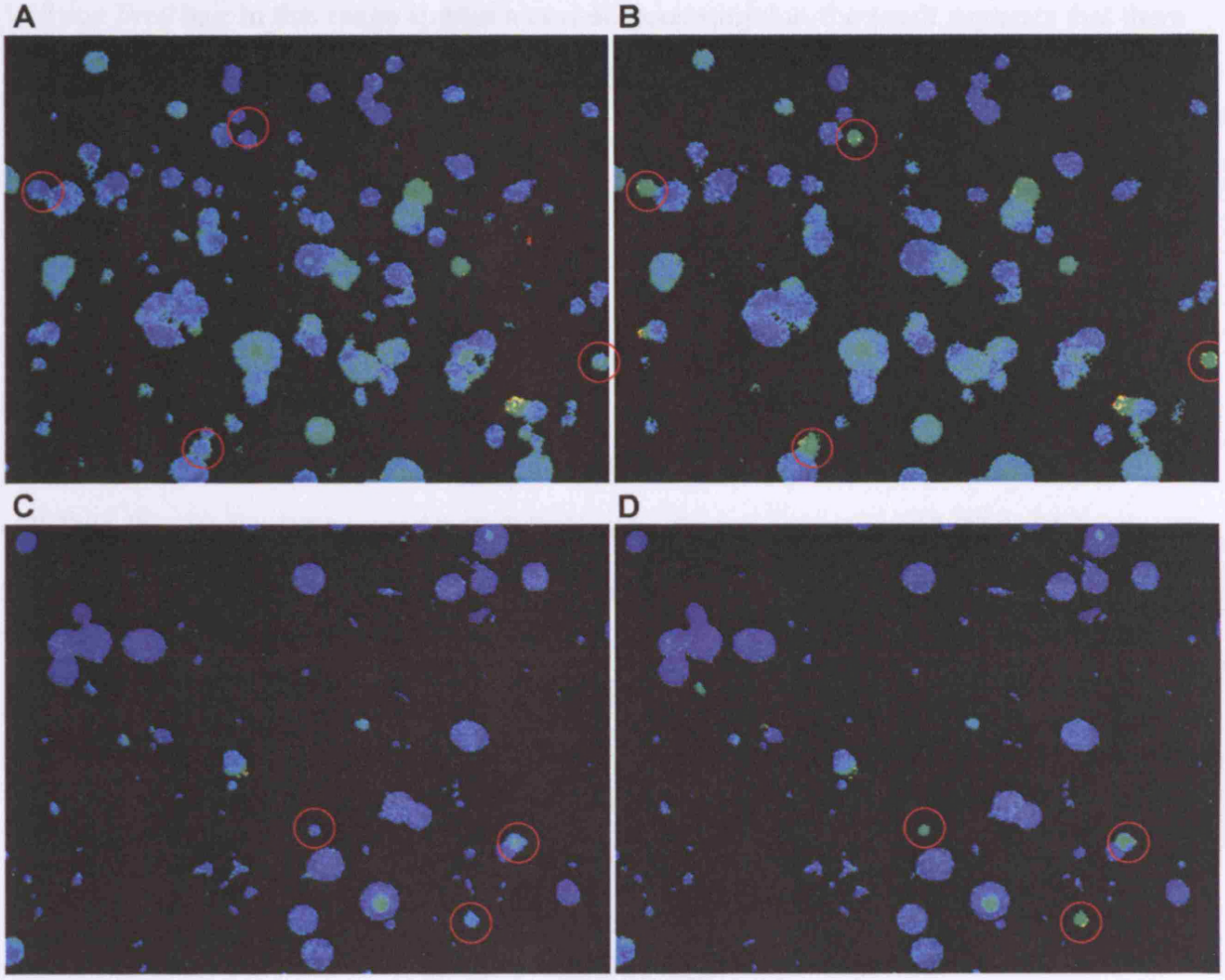

Figure 22. Calcium imaging – Menthol activation
Representative photo of the calcium imaging experiment.
DTA-control: before (a) and after (b) activation
DTA-CRE: before (c) and after (d) activation

4.2.5 Pain behaviour

4.2.5.1 Mechanical stimulation

4.2.5.1.1 von Frey filaments

The responses to mechanical stimulation using von Frey hairs were tested on 14 DTA-CRE and 13 DTA-control mice (Fig. 23A). No significant differences between the two groups were found. The DTA-CRE mice recorded a 50% threshold of 0.73±0.05g and the DTA-control mice a 50% threshold of 0.89±0.08g (P= 0.142).

As von Frey hair in this range applies a non-noxious stimulus, the result suggests that there is no difference in the non-noxious response between the DTA-CRE and DTA-control mice.

4.2.5.1.2 Randall Selitto apparatus

A noxious pressure is applied using the Randal Selitto apparatus (Fig. 23B).

The response to mechanical stimulation using the Randall Selitto apparatus was tested on 13 DTA-CRE and 8 DTA-control mice. A significant difference in the applied weight resulting in withdrawal of the tail was found, 381.67±48.65g for the DTA-CRE and 69.48±5.00g for the DTA-control, ($P<0.001$). As a further note of the 13 DTA-CRE mice, 7 reached the cut-off point of 500 g for all 3-4 measures.

The latter results suggest that noxious mechanical responses are conveyed through $Na_v1.8$ as these neurons have been ablated in the DTA-CRE mouse. The data also suggests, that the response obtained using the von Frey filaments is mediated through either Aβ-fibres or through a subset of Aδ and C-fibres not containing $Na_v1.8$.

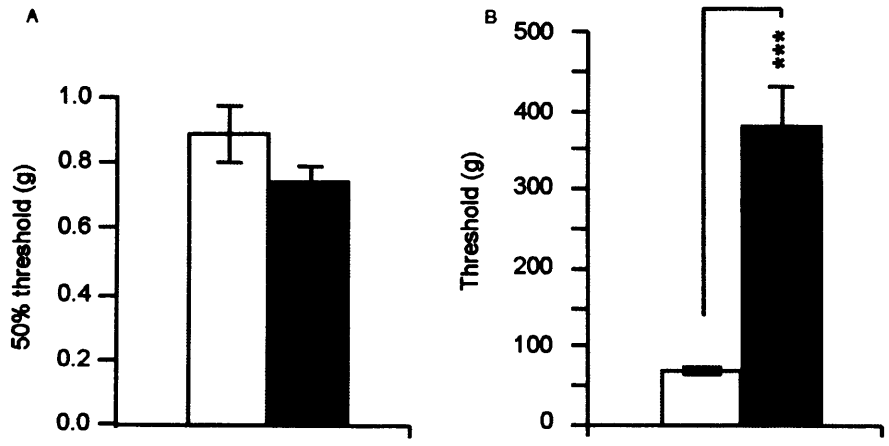

Figure 23. Mechanical responses.
A: von Frey, B: Randall Selitto
■ DTA-CRE, □ DTA-control

4.2.5.2 Cold, but not heat affected in the DTA-CRE

Two different models for testing thermal responses were used, Hargreave's instrument and the Hot plate.

Hot plate differs from Hargreave's in the nature of response. In hot plate a supra spinal response is measured whereas Hargreave's produces a withdrawal reflex. Interestingly, both thermal tests showed no differences between the DTA-CRE and DTA-control. From the immunohistochemistry and calcium imaging we know that not only are the large majority of the small sensory neurons ablated, but the $TrpV_1$, reportedly involved in acute heat sensation has also been strongly affected by the deletion. This suggests that other proteins must be involved in the transduction of heat, located either in the remaining $Na_v1.8$-negative small sensory neurons or in the Aδ/Aβ-fibres.

4.2.5.2.1 Hargreave's instrument (Fig. 24A)

The acute response to radiant heat using Hargreave's instrument was tested on 6 DTA-CRE and 7 DTA-control mice. No significant difference was found between the two groups with latencies of 8.48±0.71s for the DTA-CRE and 6.55±1.21 for the DTA-control, (P=0.22).

4.2.5.2.2 Hot plate (Fig. 24B)

Mice were tested at 50°C and 55°C. For the lower temperature a total of 10 DTA-CRE and 5 DTA-control mice were tested showing no significant difference in response, 14.42±1.43s and 14.26±1.75s respectively, (P=0.96).

At 55°C 13 DTA-CRE and 7 DTA-control mice were tested and no significant difference was found. 36.35±3.66s and 31.93±5.06s are the data for the DTA-CRE and DTA-control respectively, (P=0.49).

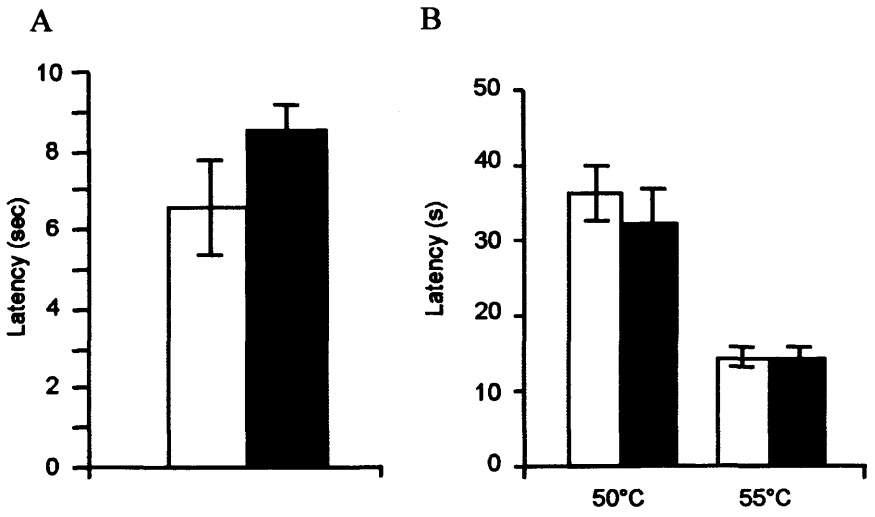

Figure 24. Thermal responses to heat.
Hargreaves' (A), Hot plate (B).
■ DTA-CRE, □ DTA-control

4.2.5.2.3 Cold plate (Fig. 25)

In total 7 DTA-CRE and 7 DTA-control mice were tested on the cold plate. A very significant phenotype was recorded for the DTA mice showing an average of 3.57±2.82 paw lifts compared with 44.743±8.71 paw lifts for the DTA-control group. ($P<0.001$).

The results show that without $Na_v1.8$-expressing neurons, the sensation of noxious cold is almost completely removed.

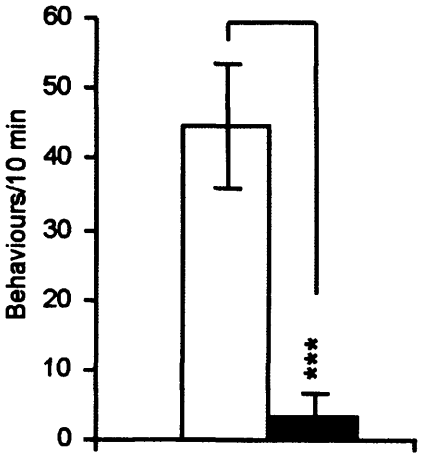

Figure 25. Responses to cold plate (0°C)
■ DTA-CRE □ DTA-control
***: $P<0.001$

4.2.5.2.4 Acetone (Fig. 26)

Acetone activates $TrpM_8$ and gives rise to a cold stimulation due to the evaporation. In total 5 DTA-CRE mice and 6 DTA-control mice were tested for responses to acetone. No difference was found between the two groups showing an average of 6.26±0.64 and 5.89±0.99 sec of licking and biting measured for 1 min.

The cold produced from the evaporation of acetone is less cold than the cold plate and suggests that a different mechanism is in place for this response involving neurons not expressing $Na_v1.8$.

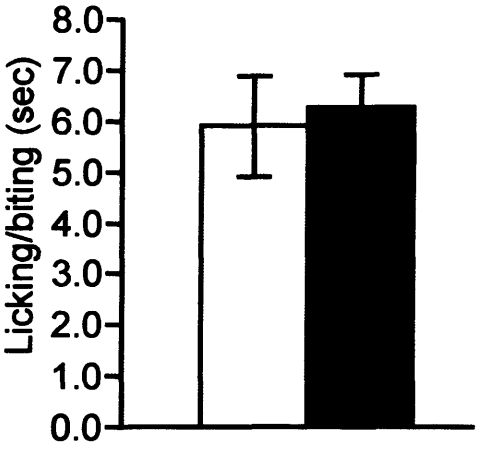

Figure 26. Responses to acetone
■ DTA-CRE □ DTA-control

4.2.5.3 Inflammatory responses dramatically reduced in DTA-CRE

Responses to inflammation are affected by the host of mediators released due to tissue damage. For this reason the DTA mouse was analysed by using different inflammatory models.

4.2.5.3.1 Formalin (Fig.27)

Responses from 8 DTA and 6 DTA-control mice were recorded and the results occur in two observable phases. For the first phase (0-10 min.), the DTA mice displayed 60.00±8.77s of pain behaviour compared with 49.00±4.50s for the DTA-control. This does not translate to any significant difference when compared using an unpaired 2-tailed t-test (P=0.25).

The results from the second phase (10-60 min.) showed a very strong difference. The DTA-CRE mice spent 55.13±26.72s on licking and biting whereas the DTA-control mice spent 197.5±20.31s (P =0.0015).

A more detailed visualisation of the behaviour is made by expressing the number of behaviours recorded over consecutive 5 minute intervals. In the second phase the DTA-control mouse behaviour peaks between 15 and 35 minutes after the formalin injection and a highly significant difference is calculated for the intervals from 15-20m (P<0.001), 20-25m (P<.001), 25-30m (P<0.001).

Very interestingly, the measurements from the first phase, reportedly a direct chemical stimulation of the sensory neurons, were not different between the DTA-CRE and the DTA-control. When looking at the data from the second phase, an almost complete ablation of responses was measured. This indicates that different neurons are involved in the different phases and most likely is the $Na_v1.8$-expressing neurons responsible for the second phase.

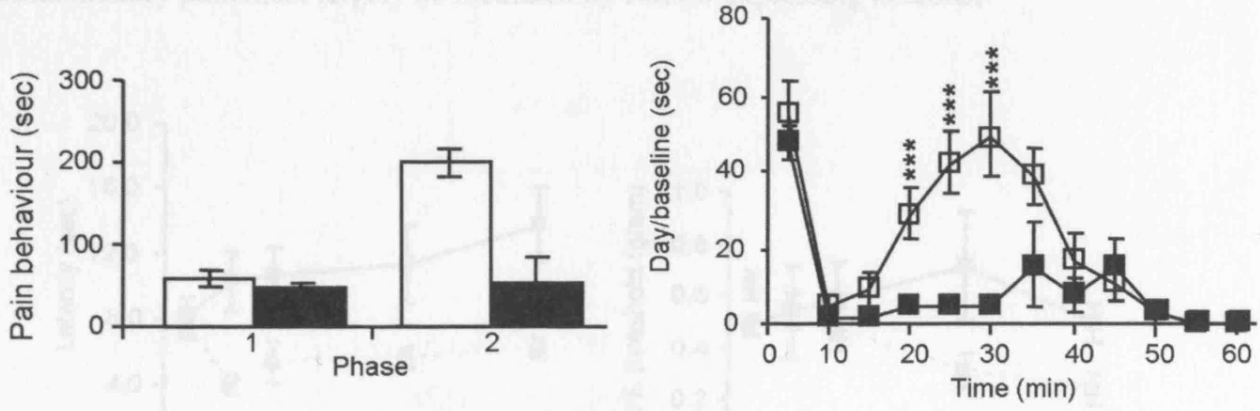

Figure 27. Responses to formalin:
■ DTA-CRE □ DTA-control
Phase 1 (chemical) and phase 2 (inflammatory) (left).
Inflammatory pain visualised over time (right).

4.2.5.3.2 Freund's Complete Adjuvant (CFA) (Fig. 28)

Thermal and mechanical responses were recorded from mice injected with CFA and compared to baseline recordings made prior to the injections. The same groups of mice, 6

DTA-CRE and 10 DTA-control mice were observed for both thermal and mechanical responses.

When exposed to thermal stimulation, a significant difference was seen for the DTA-CRE mice when compared to the DTA-control measurements, for all the days of recording except for day 2 due to variance between the DTA mice. (P-values: Day 1=0.0062, Day 2=0.103, Day 5=0.0042, Day 8<0.001).

For the DTA-control mice a much more classical hyperalgesic phenotype was recorded, a strong initial hyperalgesic response converging with the baseline level at day 8. (P-values: Day 1<0.001, Day 2=0.035, Day 5=0.009, Day 8=0.132).

For the response to the mechanical von Frey stimulation a strong difference between the DTA-CRE and the DTA-control mice was recorded for all the days measured. The DTA-CRE mice produce a hypo-algesic response (P-values: Day 1<0.001, Day 2=0.035, Day 5=0.009, Day 8=0.132), and the DTA-control develops an increased sensitivity, (P-values: Day 1= 0.001, Day 2=0.035, Day 5=0.009, Day 8=0.132).

The result confirms what was measured in the second phase of the formalin test, that inflammatory pain must largely be mediated by $Na_v1.8$-expressing neurons.

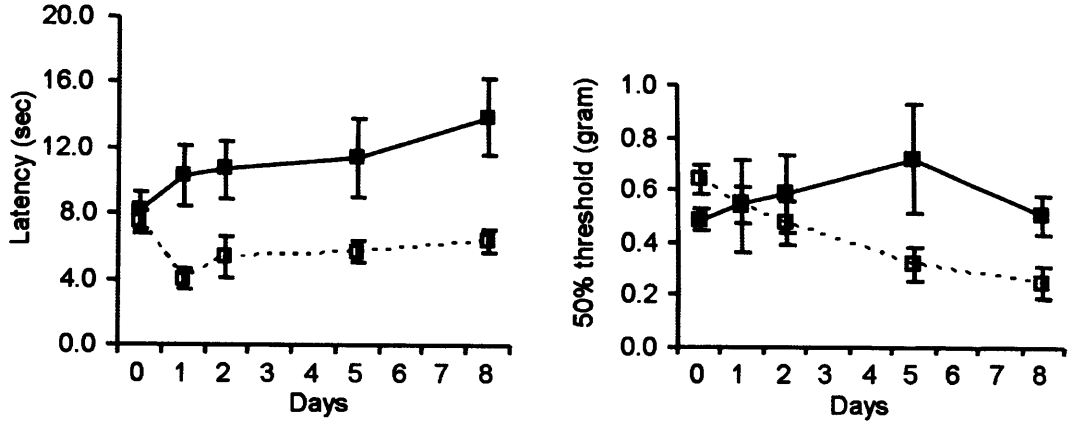

Figure 28. Responses to CFA
■ DTA-CRE □ DTA-control
Thermal responses using Hargreave's instrument (left), and mechanical responses using von Frey (right).

4.2.5.3.3 Nerve growth factor (NGF) (Fig. 29)

NGF is released after tissue damage. It activates the TrkA receptor and is also involved in the release of other compounds such as CGRP and substance P.

To test for responses to injected NGF 5 DTA-CRE and 6 DTA-control mice had NGF injected into the plantar surface of their left hind paw. A baseline for thermal responses using Hargreave's instrument was recorded prior to injections. At 20, 40 and 60 minutes post injection, and after 2, 4, 7 and 24 hours again the thermal responses were tested. The DTA mice did not develop any increased sensitivity when exposed to the heat. (P-values: 20m<0.001, 40m=0.004, 1h<0.001, 2h=0.006, 4h=0.006, 7h=0.02, 24h=0.07).
A very strong phenotype developed for the DTA-control mice with strong hyperalgesic responses lasting until the final measurement after 24h where the DTA-control mice again showed baseline responses. (P-value=0.34). (ANOVA for 20m-7h: P<0.05)

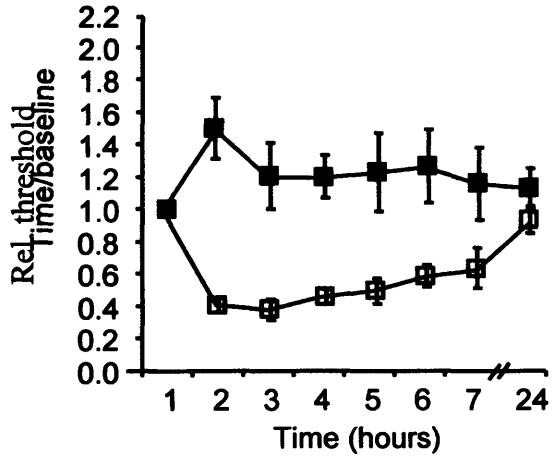

Figure 29. Thermal responses to NGF
■ DTA-CRE □ DTA-control

4.2.5.3.4 Carrageenan (Fig. 30)

Using the Hargreave's instrument thermal responses were tested before and after injection of Carrageenan into the plantar surface of 5 DTA and 6 DTA-control mice. The thermal responses were tested at 1, 2, 3, 4, 5 and 6 hours post injection. The DTA mice did not develop any significant changes in response compared with the DTA-control mice which showed a very strong increase in sensitivity to the thermal application lasting until the end

of the assay. (P-values: 30m=0.008, 1h=0.011, 2h=0.004, 3h=0.016, 4h=0.017). (ANOVA: $P<0.05$)

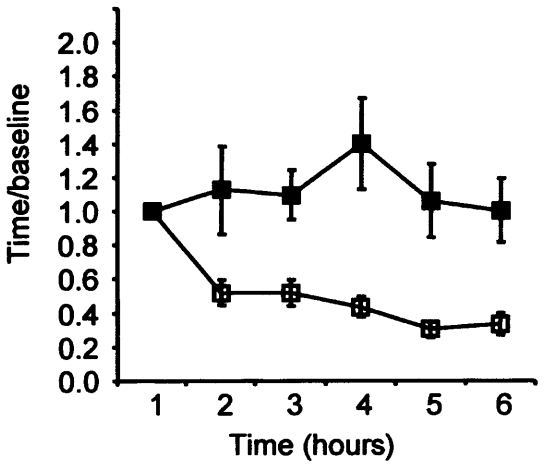

Figure 30. Thermal responses to Carrageenan
■ DTA-CRE □ DTA-control

4.2.5.3.5 Capsaicin (Fig. 31)

$TrpV_1$ is activated by Capsaicin. To test for responses to capsaicin injections of 0.5µg of capsaicin were delivered to in total 5 DTA-CRE and 5 DTA-control mice with immediate pain behaviour (licking and biting) recorded for up to 5 minutes per mouse after each injection.

The DTA-control mice showed on average of 26.67±7.14 sec compared with the DTA-CRE that showed an average of 1.33±0.93 sec pain behaviour. This suggests that the $TrpV_1$ neurons, normally responding to capsaicin, have been ablated.

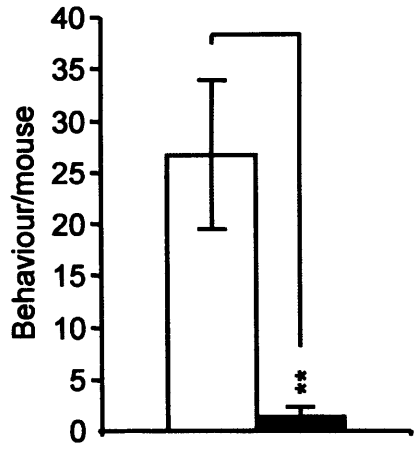

Figure 31. Behavioural responses to Capsaicin
■ DTA-CRE □ DTA-control

4.2.5.4 Spontaneous pain

The development of spontaneous pain is a phenomenon particularly experienced in different pain states. By injecting CFA into two different places an inflammatory response is generated giving rise to spontaneous pain which can be assessed by observing the mouse and count numbers of paw-lifts (Fig. 32).

On day 0, 20 μL of CFA was injected into the plantar surface and to the side of the knee in 8 DTA-CRE and 5 DTA-control and observations made on day 1, 2 and 7.
A strong significant difference was observed on day 1 with the DTA-CRE mice showing an average of 2.0±0.78 spontaneous paw lifts compared with the DTA-control mice showing 9.6±1.06 paw lifts. (P-value< 0.001).
No significant difference was observed from either day 2 (DTA-CRE: 1.0±0.42, DTA-control: 1.8±0.73, P-value=0.323) or day 7 (DTA-CRE: 0.13±0.13, DTA-control: 0.40±0.34, P-value=0.399).

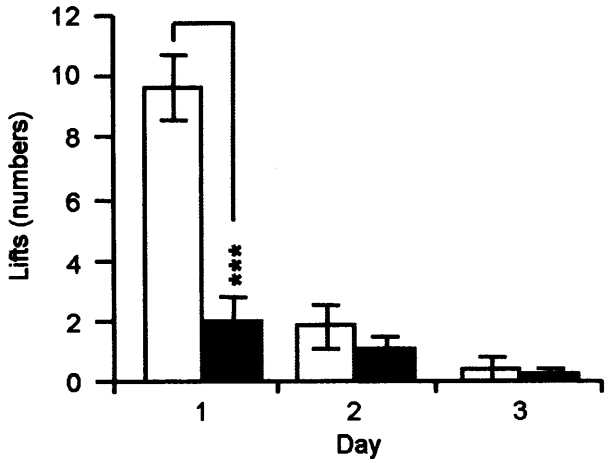

Figure 32. Spontaneous pain behaviour.
■ DTA-CRE □ DTA-control

4.2.5.5 Neuropathic pain

Together 13 DTA-control and 8 DTA-CRE mice underwent Chung surgery to induce neuropathic pain. Pre-surgery baselines had been measured for responses to mechanical (von Frey) and thermal (Hargreaves' instrument) stimulation. The mice were tested for mechanical hyperalgesia using von Frey on days 3, 5, 7, 10, 13, 17, 24 and 31 (Fig 33A). The mice were tested further for thermal hyperalgesia on days 4, 11, 18, 25 and 31 (Fig. 33B).

Responses to von Frey filaments and Hargreaves' instrument were not significantly different between the DTA and the DTA-control mice. Using a two way measured ANOVA no difference was calculated over the time interval for both responses to von Frey filaments and Hargreaves' instrument ($P>0.05$). The results suggest that neuropathic pain affects other sensory neurons than $Na_v1.8$-expressing.

von Frey	
Day	P-value
3	0.32205
5	0.207492
7	0.938007
10	0.739494
13	0.433298
17	0.057228
24	0.504248
31	0.444475

Hargreaves'	
Day	P-value
4	0.4957704
11	0.6170667
18	0.6739001
25	0.5743557
31	0.7224760

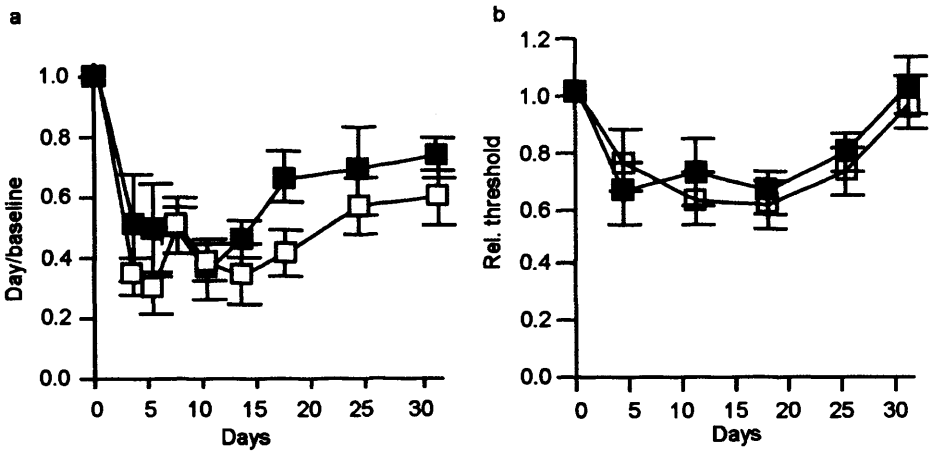

Figure 33. Responses to Neuropathic pain.
■ DTA-CRE □ DTA-control
von Frey filaments (left) and thermal responses (right).

4.2.6 Single unit electrophysiology

The following data was collected by Mr. Curtis Asante.

Electrophysiology from the deep dorsal horn of the spinal cord allows the investigation and characterisation of wide dynamic range neurons activity after peripheral stimulation.

Peripheral nerves can be stimulated in the following two way to gather different data. Electrical stimuli were used to investigate thresholds and prolonged stimulation. Physical stimuli such as pinch, brushing etc. were applied to mimic the thermal or mechanical stimulation used when assessing the behavioural responses.

Recordings were made from 17 Wide Dynamic Range (WDR) neurons, (DTA-CRE: n=8, DTA-control: n=9) with a mean depth of 517.5±29μm for the DTA- and 471.3±36μm for DTA-control, a depth equivalent with the deep laminae of the dorsal horn (Fig. 34 left).

The electrical thresholds for A-fibres were not different between the DTA-CRE and DTA-control, but a strong significant difference was measured for the C-fibre threshold ($P<0.05$) (Fig. 34 right)

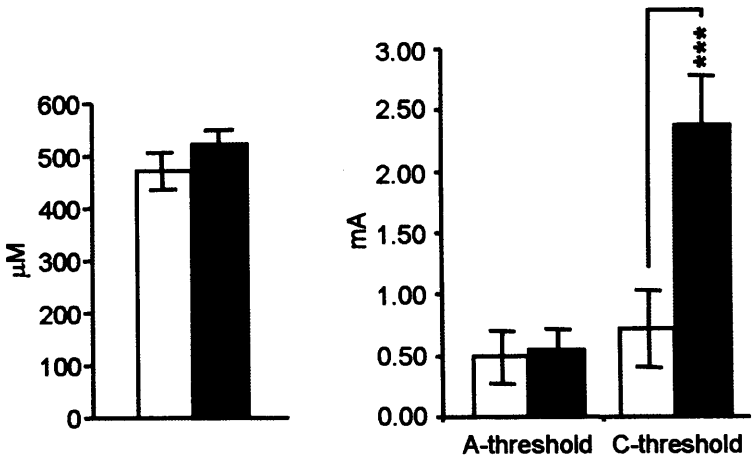

Figure 34. Electrical stimulation of WDR neurons
Depth of electrode (left)
A-and C-fibre thresholds to electrical stimulation (right)
■ DTA-CRE □ DTA-control

Following identifying the thresholds from electrical stimuli a number of noxious stimuli were applied and single-unit responses recorded.

Strong deficits in the responses to noxious mechanical stimulation of the paw were measured for the DTA mice when exposed to both pinch ($P<0.05$) and von Frey filaments>1 gram ($P<0.05$ for filament 15g, 26g and 60g) (Fig. 35). In contrast when lighter mechanical stimuli were applied using a brush, no differences were recorded between the DTA-CRE and the DTA-control mice ($P=0.839$). The results confirm the general division of noxious information being conveyed through small sensory neurons, largely ablated in the DTA-CRE mouse and non-noxious information largely conveyed through the large sensory neurons, remaining in the DTA-CRE-mouse.

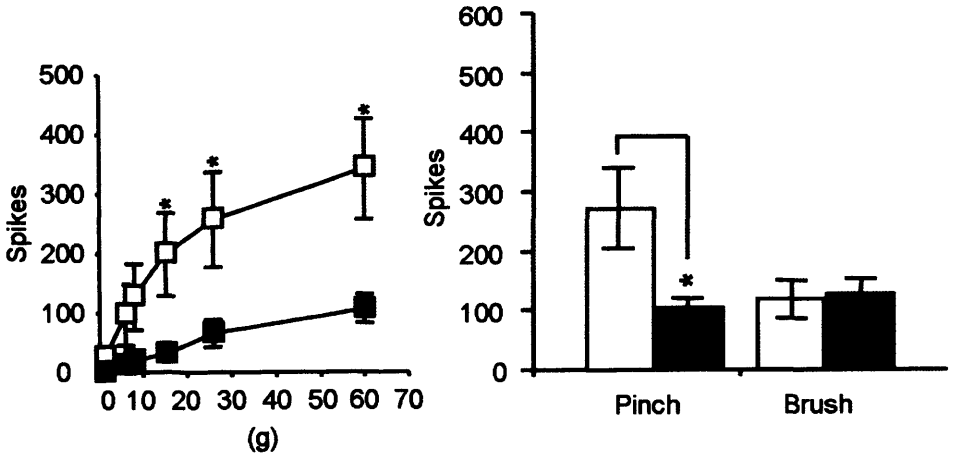

Figure 35. Spikes generated after mechanical stimulation.
von Frey filaments (left)
Pinch and brush (right)
■ DTA □ DTA-control

When water at different temperatures (1°C-50°C) (Fig. 36) was applied to the paw no significant differences were found between the DTA-CRE and DTA-control mice. The data compliments the behavioural data obtained from Hargreave's instrument and the Hot plate.

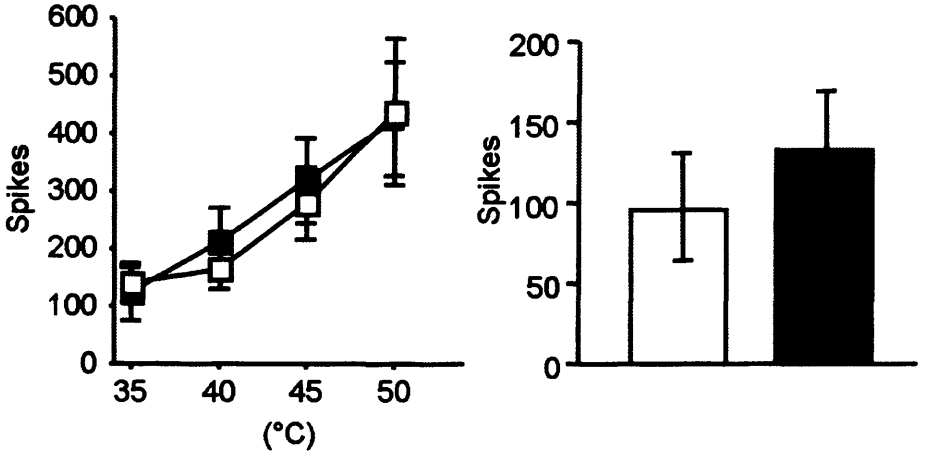

Figure 36. Spikes generated after thermal responses
Warm temperatures (left)
Cold (1°C) (right)
■ DTA-CRE □ DTA-control

After administering a train of electrical stimuli – 16 stimuli at 3x threshold – the response evoked was divided on the basis of conduction velocity and generated spikes and used to

investigate post-discharge and wind up. Wind up – the total spikes after 16 stimuli minus input which is the number of spikes from one stimuli multiplied by 16.
An accumulation of the post-discharge after each of the 16 stimuli showed a significant difference indicating that less activity was conveyed. For both phenomenons the DTA-CRE mice showed a significantly reduced response when compared with the DTA-control mice.

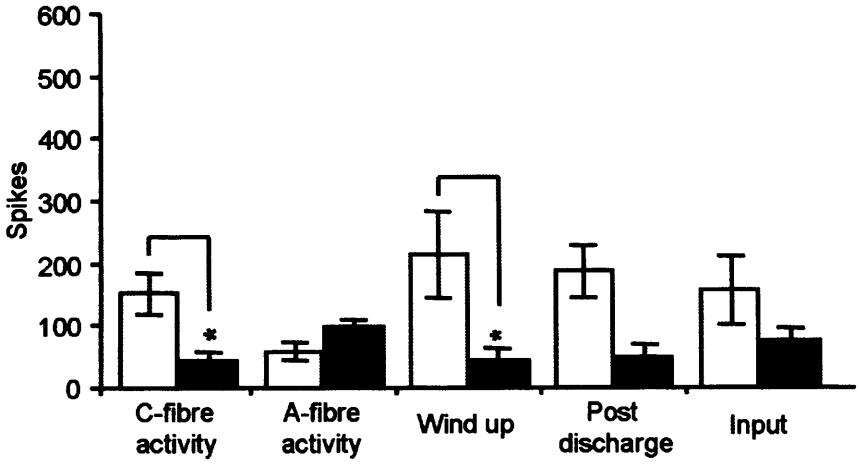

Figure 37. Electrophysiology recordings from WDR neurons
From left to right; C-fibre activity ($P<0.05$), A-fibre activity ($P=0.044$), Wind up ($P=0.043$), post-discharge ($P=0.0106$) and input ($P=0.204$).
■ DTA-CRE □ DTA-control

4.2.7 Microarray

From the investigation of the regulation of transcripts between the DTA-CRE and DTA-control using microarray it is possible to identify possible transducers involved in different pain modalities. If a transcript is strongly downregulated it suggests involvement in the transduction of noxious mechanical pain, inflammatory pain or cold pain. Likewise for the upregulated transcripts, these might be responsible for some of the remaining modalities such as thermal pain and light mechanical sensation.

DRG from 6 male DTA-control and 6 male DTA-CRE mice were dissected and the total RNA was extracted and pooled together in pairs giving, in total, 3 DTA-control samples and 3 DTA-CRE samples, one pair for each gene chip. From each mouse, one DRG cell body was sectioned and stained with anti-peripherin and anti-neurofilament to double-

check the genotype. For certainty, a second PCR genotyping procedure was carried out and a neurofilament/peripherin staining. This was performed as a precursor to the microarray as the microarray experiment is very expensive. Both the second genotyping and the immunohistochemistry confirmed the genotype was correct.

The amount of total RNA was tested on a 1.2% agarose gel before the first and second strand cDNA synthesis, cRNA synthesis and biotin labelling were performed and quantified. Finally the labelled cRNA was fragmented before it was loaded on gene chips.

4.2.7.1 Total RNA

Sample	ng/µL	260/280	260/230
DTA-control	752.76	2.09	2.29
DTA-control	1148.5	2.07	2.28
DTA-control	972.31	2.09	2.29
DTA-CRE	177.12	2.05	2.28
DTA-CRE	252.54	2.05	2.06
DTA-CRE	277.96	2.05	1.80

4.2.7.2 cRNA

Sample	ng/µL	260/280	260/230
DTA-control	152.34	1.95	2.63
DTA-control	141.78	1.95	2.57
DTA-control	132.02	1.96	2.26
DTA-CRE	126.07	1.98	1.68
DTA-CRE	165.04	1.98	2.39
DTA-CRE	130.13	1.95	2.32

Figure 38. Control of Fragmentation
Biotin labelled RNA was fragmentated and checked on a mini-gel.
Lane 1: Marker
Lane 2: DTA-CRE
Lane 3: DTA-control

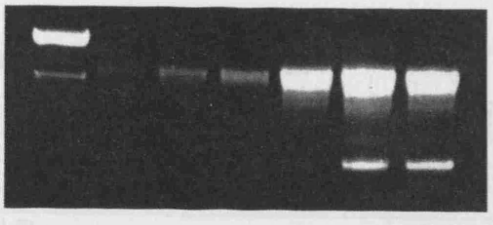

Figure 39. Total RNA
From left; Lane 1: size marker.
Lane 2-4 DTA-CRE
Lane 5-7 DTA-control.

Finally, the hybridisation, scanning and analysis were performed by the Scientific Support Services, Wolfson Institute for Biomedical Research, UCL, UK.

4.2.7.3 Regulation of several genes after deletion of nociceptors

From a list of around 10,000 genes with a P-value<0.05 a list with the largest gene changes was generated, categorising the genes into groups depending on their functionality; Sodium channels, Calcium channels, Trp-channels etc.

Voltage Gated Sodium Channels

Gene	ID	Fold change	P-value
Na_v 1.1	Scn1a	+1.8	3.99E-05
Na_v type III, beta subunit	Scn3b	-2.9	4.75E-08
Na_v 1.7	Scn9a	-2.2	2.03E-05
Na_v type II, beta subunit	Scn2b	-3.7	6.59E-04
Na_v 1.8	Scn10a	-37.6	5.96E-07
Na_v 1.9	Scn11a	-23.32	5.04E-08

Voltage Gated Calcium Channels

Gene	ID	Fold change	P-value
Beta-3 subunit	Cacn1b	-1.56	1.18E-05
Beta-3 subunit	Cacn3b	-1.5	0.000061
L-type Alphe-1C subunit	Cacna1c	-2.96	1.01E-6
N-type Alpha 1B subunit	Cacna1b	-3.18	1.18E-06
α2δ -1 subunit	Cacna2d1	-3.38	1.14E-06

Trp-Channels

Gene	ID	Fold change	P-value
$TrpV_1$	Trpv1	-3.5	4.66E-06
$TrpC_3$	Trpc1	-6.4	1.22E-08
$TrpC_6$	Trpc3	-8.6	9.57E-09
$TrpA_1$	Trpa1	-13.5	5.01E-05

Growth factor receptors

Gene	ID	Fold change	P-value
cRET	Ret	-1.47	-1.86E-05
TrkC	Ntrk3	+1.3	0.008
TrkB	Ntrk2	+1.25	0.00098
NGF receptor	Ngfr	-1.15	0.015
TrkA	Ntrk1	-8.9	9.83E-06

GDNF receptor alpha1	Gfra4	+1.2	0.002
GDNF receptor alpha2	Gfra2	-4.9	2.62E-09
GDNF receptor alpha3	Gfra3	-2.7	3.56E-05

Transcription factors

Gene	ID	Fold change	P-value
Runx1	Runx1	-4.1	9.27E-08
Runx3	Runx3	+2.37	1.57E-06

Neuropeptides

Gene	ID	Fold change	P-value
BDNF	Bdnf	-1.8	0.00051
Substance P	Tac1	-2.87	8.22E-06
CGRP beta	Calcb	-3.6	1.02E-07
CGRP alpha	Calca	-4.26	0.00076

Kinases

Gene	ID	Fold change	P-value
PKA		-3.07	
PKA regulatory subunit 2		-2.6	
PKA anchoring subunit 2		-3.98	3.92E-06
PCK delta		-9.39	8.99E-08
PCK theta		-8.14	7.46E-07

Others

Gene	ID	Fold change	P-value
P_2X_3	P2rx3	-11.93	7.10E-10
5-HT_4	Htr4	-15.96	1.41E-08
NPY-Y2 receptor	Npy2r	-20.9	9.79E-08
Edg7 LPA receptor	Edg7	-43.8	3.33E-12
MrgA2	Mrgpra2	-10.77	2.75E-07
MrgA3	Mrgpra3	-18.03	5.44E-06

Table 1. Altered transcripts in DRG from the DTA-KO.

4.2.8 Quantitative RT-PCR confirming microarray data

Total RNA from the same mice used for the microarray study, as well as total RNA from a new set of mice (DTA-CRE and DTA-control), was converted to cDNA using reverse transcriptase and used for qRT-PCR. β-Actin was used to normalise the data.

A complete deletion of $Na_v1.8$ was expected and primers for qPCR of $Na_v1.8$ and four other regulated genes, Edg7, $TrpC_6$, $TrpV_1$ and $TrpM_8$ were constructed.

After normalisation, the level of mRNA for $Na_v1.8$ in the DTA-CRE mouse was only 1.68±0.69% compared with mRNA from DTA-control mice. For the $TrpC_6$, mRNA levels were down to 4.33±0.58% and Edg7 were down to 3.35±0.42% compared with DTA-control. $TrpV_1$ levels in the DTA-CRE mouse were 28.4±3.63% of DTA-control levels and no significant difference between DTA-CRE and DTA-control were found for $TrpM_8$ mRNA levels.

As mentioned, two different cDNA preparations were used. The first utilised the original RNA extracted and used for the microarray experiment. The second preparation was prepared later to test if there would be any difference between the first and a later preparation. For the qRT-PCR both cDNA preparations were used and there was no difference between them, validating the first RNA extraction which was used for the microarray experiment.

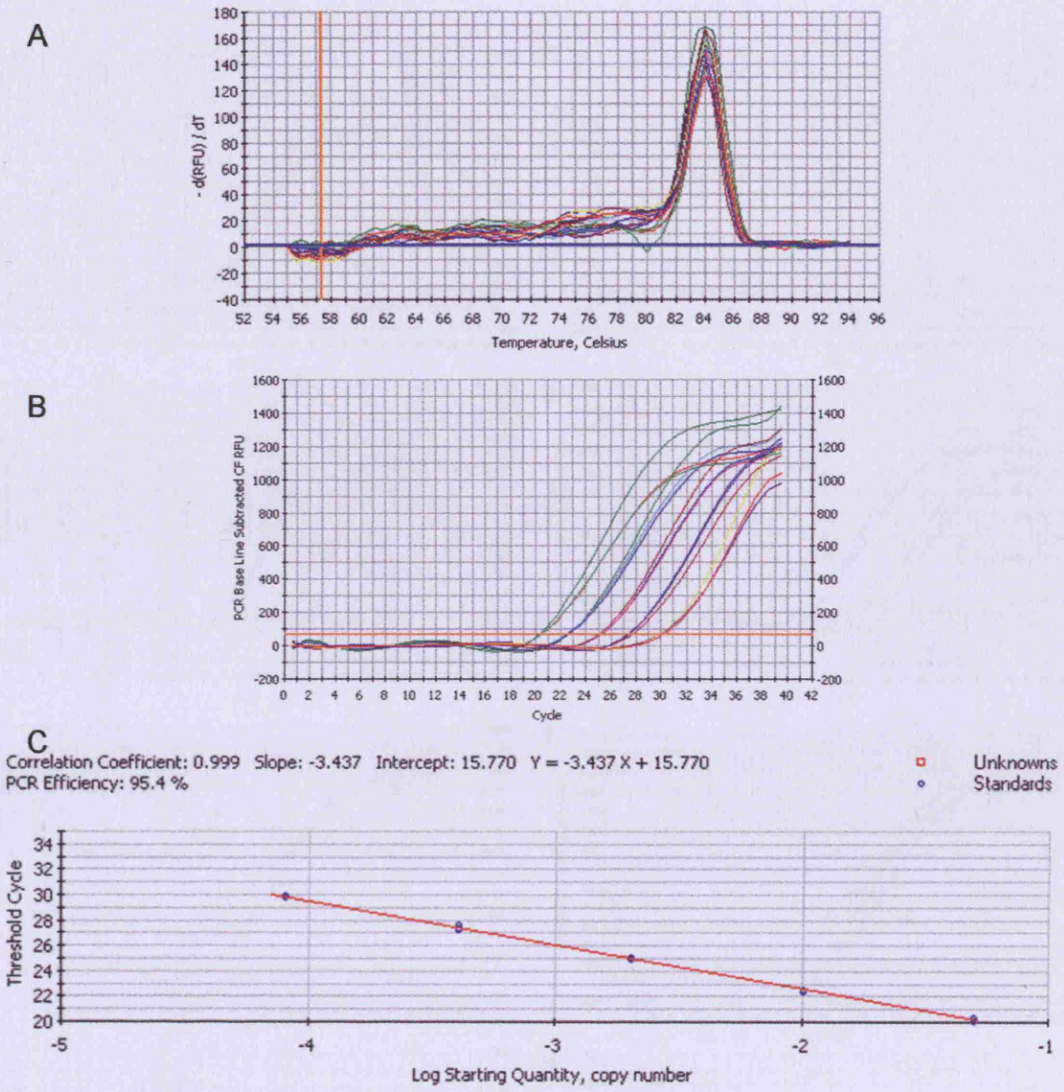

Figure 40. An example for testing primers for qRT-PCR
A: Melting curve for the primers for β-Actin
B: PCR quantification for standard curve
C: PCR standard curve

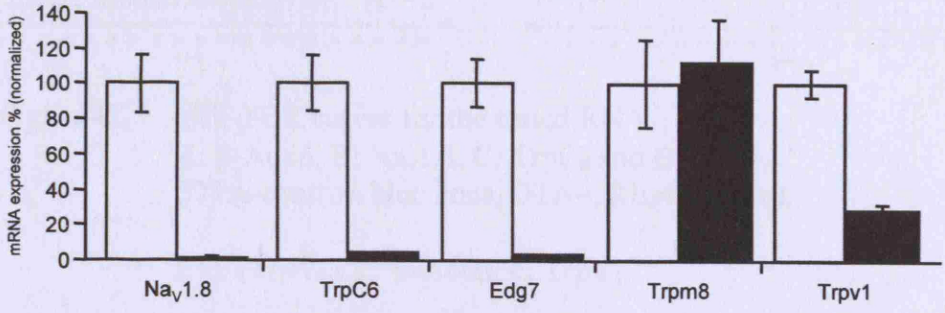

Figure 41. Quantitative RT-PCR.
qRT-PCR were tested for $Na_v1.8$ $TrpC_6$ and Edg7, $TrpM_8$ and $TrpV_1$.
■ DTA-CRE □ DTA-control

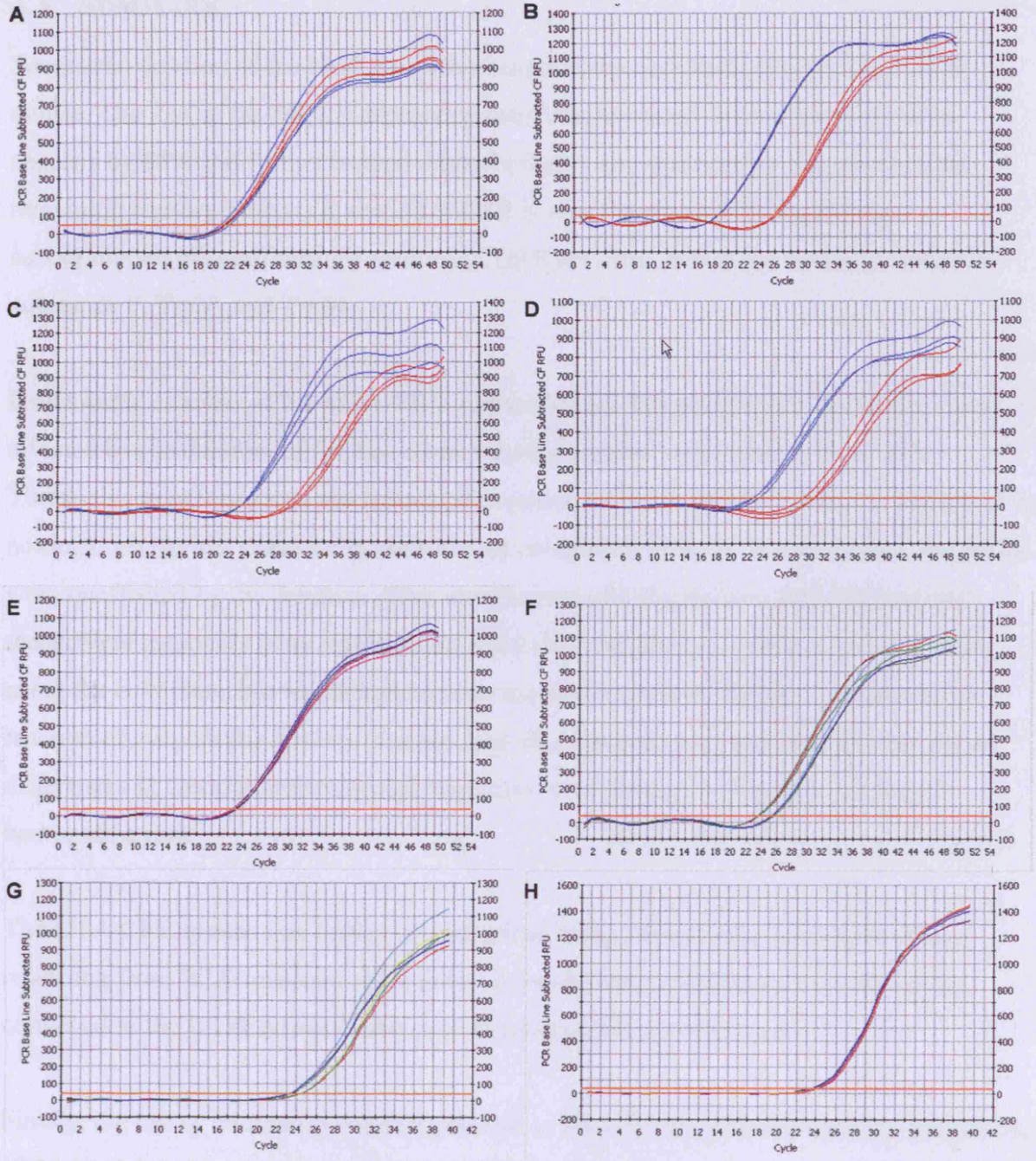

Figure 42. qRT-PCR curves for the tested RNA.
A: β-Actin, B: $Na_v1.8$, C: $TrpC_6$ and D: Edg7
(DTA-control: blue lines, DTA-CRE, red lines).

E+F ($TrpV_1$): E: β-Actin, F: $TrpV_1$.

G+H ($TrpM_8$): G: β-Actin, H: $TrpM_8$.

4.3 SUMMARY

Successful deletion of the $Na_v1.8$ positive neurons was obtained using the $Na_v1.8$Cre mouse. The loss of the $Na_v1.8$-expressing neurons decreased the peripherin positive neurons by 88% and further immunohistochemistry and calcium imaging showed the remaining sensory neurons in the DTA-CRE mouse belonged to the peptidergic subpopulation of small sensory neurons (CGRP positive) with some testing positive for substance P, $TrpV_1$ and $TrpM_8$.

Behavioural analysis of the DTA-CRE mouse showed that several pain modalities were affected by the deletion of the $Na_v1.8$-expressing neurons – see table 2, page 102. Thresholds to non-noxious mechanical stimulation were not affected, whereas responses to noxious mechanical stimulation were almost completely ablated. Thermal acute thresholds were unaffected by the deletion. After introduction of inflammation both thermal and mechanical responses were significantly reduced. Responses to cold were ablated when using the cold plate, but no difference were measured when cold from the endothermic reaction of evaporating acetone was invoked. Spontaneous pain was also ablated, but no differences in thermal or mechanical responses were measured after introduction of neuropathic pain

The DTA-CRE mouse was further characterized using extracellular electrophysiology recording from WDR neurons at the dorsal horn showing a significant loss of activity conveyed to the WDR neurons after peripheral stimulations of the paw.

Finally, the use of microarray made it possible to identify the genes affected by the deletion of the $Na_v1.8$-expressing neurons. Among the regulated genes were several already reported to be involved in some of the affected pain modalities, noxious mechanical mechanosensation, inflammatory pain and cold pain. A number of genes, not previously reported to be involved in nociception were strongly downregulated suggesting that they possibly also could be involved in some of the modalities that were lost.

Modality	DTA-KO
Cold pain	Lost
Mechanical pain	Lost
Inflammatory thermal pain	Lost
Inflammatory mechanical pain	Lost
Spontaneous pain after inflammation	Lost
Thermal pain	Retained
Neuropathic thermal pain	Retained
Neuropathic mechanical pain	Retained

Table 2. Modality characterisation of the DTA-CRE mouse

A number of pain modalities were lost whereas other remained in the DTA-CRE mouse

5 PAPIN AFFECTS THE SENSATION OF TOUCH

5.1 INTRODUCTION TO TOUCH

The ability to sense touch is vital in life – nevertheless, it is still not completely understood how touch - light mechanical stimulus - is translated into an electrical signal for transmission to the brain.

Mechanoreceptor terminals are different from those responding to noxious mechanical stimuli as they are closely associated with specialised end organs and not free nerve endings. There are four classes of mechanoreceptors generating different currents depending on their end organ; Merkel cells, Meissner cells, Pacinian cells and Ruffini corpuscle – see fig. 43.

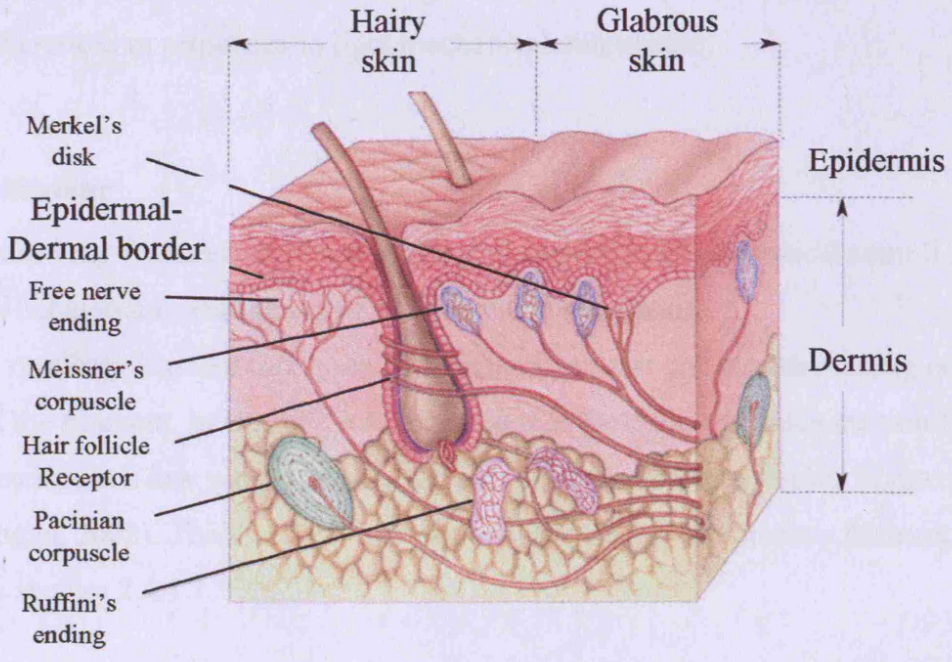

Figure 43. Overview of the skin layers
Free nerve endings responding to noxious insults and end organs responding to light mechanical stimulation.

Merkel cells	slow adapting type 1 (SA1) responsive to low frequency vibration and low intensity constant pressure.
Meissner corpuscles	rapidly ad#apting and responsive to skin deformation which triggers

	a train of action potentials that will ease quickly and recommence again when the deformation returns to normal.
Pacinian corpuscles	very rapidly adapting to indentation of the skin, they can detect changes in strength of the stimuli.
Ruffini corpuscles	detect skin stretch. Their response is slowly adapting type 2 (SA2), adapting more slowly that than Merkel cells.

Table 3. Mechanoreceptors

Each class has different response properties and patterns of action potentials depending on their adaptation to stimulation

5.1.1 Investigation of mechanosensation

In general, there are not many ways to investigate mechanisms of touch as it is a very subtle response. *In vitro*, different electrophysiology techniques have been used. *In vivo* testing is based on differences in responses to light mechanical stimulation.

5.1.1.1 Behaviour

The use of von Frey filaments to test sensitivity to non-noxious mechanical stimuli is known from behavioural assessment of mechanical hyperalgesia.
The graded von Frey filaments apply an increasing punctuate pressure depending on the thickness of the filament. In general, it is accepted that for testing on mice the von Frey filaments bending at a low weight applied are non-noxious, whereas thicker filaments are noxious (Hogan, 2002). The assembly and protocol for the use of von Frey filaments are described in section 2.4.1.1.

Another more recent and innovative test is the 'sand paper test'. The commercially available MoTil system (TSE Systems, Germany) uses infrared tracking of the mouse to monitor the movements of the mouse in a small enclosed area. Inside the cage different tactile cues (sandpaper of different grades) have placed. The data from WT mice showed that they were able to recognise different tactile cues and spend more time on the rough than on the smooth area. A transgenic mouse line, the stomatin null mouse spent the same

amount of time on both the rough and the smooth area suggesting that the mice could not distinguish between the differences in surface texture (Wetzel et al., 2007).

5.1.1.2 Electrophysiology

The skin-nerve preparation described by (Reeh, 1988)) makes recordings from teased single nerves possible using nerve fibres from the saphenous nerve. In this set-up the nerve is still attached to the skin and physical or electrical stimulation can be applied.
Electrophysiological recordings from cultured DRG neurons have been reported by a number of groups. The techniques of mechanical stimulation of the cells have varied between groups, but in general either a pressure or a physical glass probe has been applied (Drew *et al.*, 2002;Cho *et al.*, 2002).

5.1.2 Proposed mechanisms for touch

Several mechanisms for mechanotransduction have been hypothesised over recent years. Stretch activation, where changes in the lipid bilayer stretch ion channels to change conformation; or the tethered model, where links between the extracellular or cytoskeleton proteins and the ion channels conduct the force for a conformational change; thirdly, an indirect activation model where a second messenger protein generates a signal that enforces the conformational change (Kung, 2005).

In the epidermis several cell types contribute to provide a physical barrier of the body, but they could also perform other functions. The Keratinocytes are the predominant cell type and different hypotheses have been suggested for their involvement in mechanotransduction. Merkel cells are another component of skin and they are known to be part of a complex that mediates a subset of responses to touch, but there is so far no direct evidence that merkel cells are activated by touch (Haeberle et al., 2004).

5.1.3 Papin

In a yeast two hybrid screening using the neural Plakophilin-related A*rmadillo* repeat Protein (NPRAP)/δ-catenin as bait the Plakophilin-related *Armadillo* repeat Protein-Interacting PDZ protein (PAPIN) was identified (300kDa) (Deguchi *et al.*, 2000). The PDZ

domain is a common structural domain of 80-90 amino-acid found in signalling proteins and helps anchor transmembrane proteins to the cytoskeleton and hold together signalling complexes (Harris and Lim, 2001). The *armadillo* repeat is a repeated motif of about 40 amino acids found in a number of proteins such as β-catenin, plakoglobin, adenomatous polyposis coli gene product, a regulatory protein for, a small G protein known as smg GDP dissociation stimulator and smg GDP-dissociation stimulator-associated protein. Papin distributed ubiquitously and co-localised with p0071 in epithelial cells. The p0071-protein also has *armadillo* repeats and is believed to play a role in organization of cell-cell junctions (Deguchi *et al.*, 2000). Papin has four PDZ domains in the N-terminal region and two PDZ domains in the C-terminal region. The Papin protein is processed by proteolytic cleavage to generate a secreted peptide containing two PDZ domains (Yeung et al., 2003). (Papin has since then been renamed as PDZD2 (PDZ-domain-containing 2).

A paper suggests that Papin plays a role in the regulation of the glucose homeostasis affecting the development of diabetes. In the Papin knock out mouse increased levels of insulin were observed after injections of glucose, suggesting that Papin is required for normal glucose-stimulated insulin secretion (Tsang et al., 2008)

Papin's possible relation to nociception was originally discovered after using the intracellular domains of $Na_v1.8$ as bait in a yeast-two-hybrid screening. Papin was, together with several other proteins such as annexin II light chain (P11), identified as an $Na_v1.8$ interacting proteins (Malik-Hall *et al.*, 2003). Previously, it had been shown that $Na_v1.8$ expressed in cells on its own did not express strongly in the membrane, but adding P11 proved to increase the functional expression of $Na_v1.8$ (Okuse et al., 2002). A tissue-specific mouse line deleting P11 in the $Na_v1.8$-expressing neurons was generated and tested in different pain models. For the P11 KO mouse no highly significant nociceptive phenotype were measured for inflammatory pain of acute thermal sensation., but a significant, but not substantial change to mechanical and neuropathic pain was recorded (Foulkes *et al.*, 2006).

The functional effect of Papin on $Na_v1.8$ currents were investigated by Dr. Kenji Okuse's lab. They found that by using microinjections of siRNA to knock down Papin in cultured

DRG neurons, the $Na_v1.8$ current was reduced by more than 50% (unpublished data from Kenji Okuse).

5.1.4 The Papin Knockout mouse

A Papin knock out mouse was recently generated by researchers at Hong Kong University. The mouse was generated using an embryonic stem cell line with the PDZD2 (Papin) locus disrupted. The cell line was produced using the genetrap technique ROSAFARY.
Using this technique, genes were randomly disrupted when a promoterless reporter was introduced into embryonic stem cells to tag and mutate genes randomly (Chen et al., 2004). Dr. Kwok-Min Yao, Hong Kong University, obtained the stem cell line and injected them into blastocyts for the generation of chimeras The disruption of PDZD2 transcription was confirmed by RT-PCR (Tsang et al., 2008) (Unpublished).

5.1.4.1 Analysing the Papin null mouse

In the following, the Papin null mouse will be referred to as PAPIN-KO and the Papin control littermates will be referred to as PAPIN-control.
The PAPIN-KO/control mice were generated by crossing heterozygous mice to generate a 25:50:25 ratios of PAPIN-KO, PAPIN-heterozygous and PAPIN-control. By using the breeding regime described, both PAPIN-KO and PAPIN-control mice were generated from the same parents.

5.1.4.1.1 Electrophysiology

Electrophysiological recordings of DRG were made to investigate changes in currents after mechanical displacement using whole cell patch clamping as described.

5.1.4.1.2 Immunohistochemistry

To investigate any differences in the pattern of cells in DRG, immunohistochemistry was used to identify the myelinated cells using anti-neurofilament and the small cells using anti-peripherin.

5.1.4.1.3 Behaviour

For behavioural assessment the Papin mouse was investigated with the hypothesis that the PAPIN-KO mouse might exhibit different pain thresholds compared with the PAPIN-control mouse. Responses to mechanical stimuli using von Frey filaments and the Randal Selitto apparatus were tested. For responses to thermal stimuli, the Hargreave's instrument was used, and finally formalin injected to assess inflammatory responses.

5.2 RESULTS

5.2.1 Genotyping of PAPIN mice

DNA extraction, PCR and gel electrophoresis were used to genotype the litters from PAPIN heterozygous breeding pairs (Fig. 44).

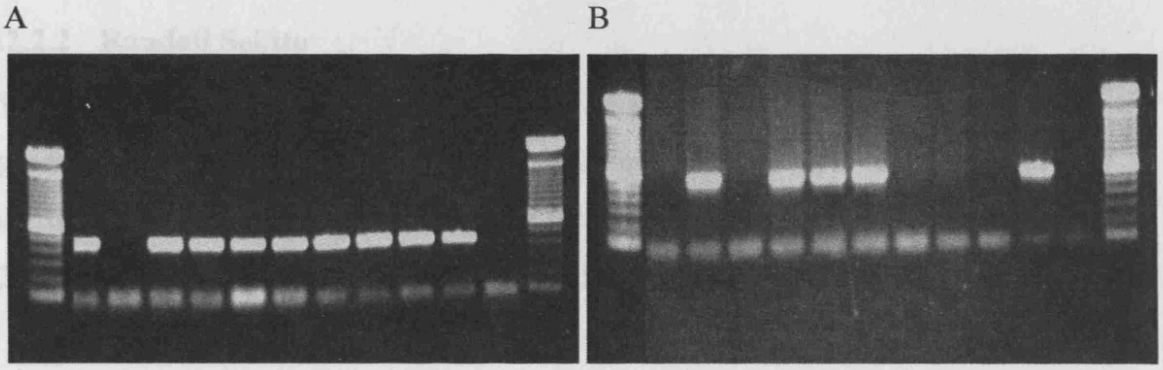

Figure 44. Genotyping the PAPIN litters.
PAPIN-control (WT) band at 420 bp. (A)
PAPIN-KO PCR with positive bands at 570 bp. (B)

5.2.2 The PAPIN-KO mouse shows increased thresholds to light touch

5.2.2.1 von Frey

The response to mechanical stimulation using von Frey hairs were tested on 9 PAPIN-KO and 12 PAPIN-control mice (Fig. 45). A highly significant difference was recorded. The PAPIN-KO mice had a 50% threshold of 1.55±0.17g compared with the PAPIN-control which exhibited a 50% threshold of 0.60±0.11g ($P<0.001$).

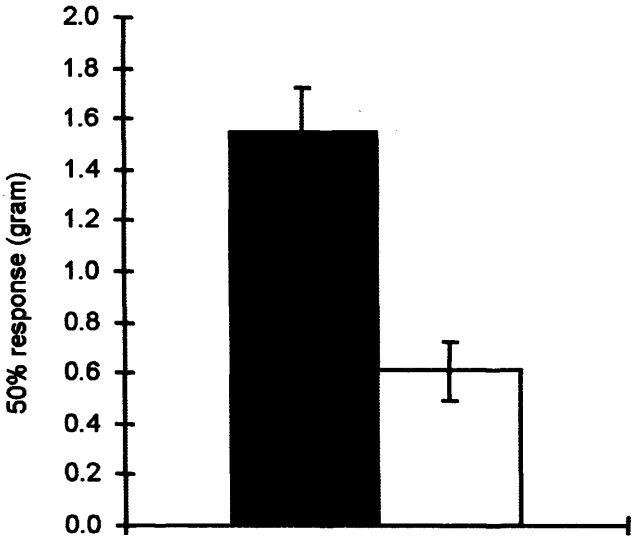

Figure 45. von Frey thresholds.
■ PAPIN-KO, □ PAPIN-control

5.2.2.2 Randall Selitto

The response to noxious mechanical stimulation was also tested using the Randall Selitto apparatus (Fig. 46). 6 PAPIN-KO and 6 PAPIN-control mice were tested without any significant difference found. The response for the PAPIN-KO was 113±11.64g and 128.33±12.07g for the PAPIN-control (P>0.05).

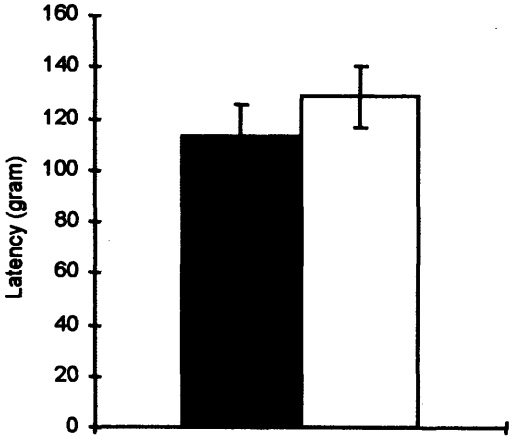

Figure 46. Acute response to noxious pressure
■ PAPIN-KO, □ PAPIN-control

5.2.2.3 Hargreave's

Responses to thermal stimulation were tested using the Hargreave's instrument (Fig. 47). In total 10 PAPIN-KO and 14 PAPIN-control mice were tested without finding any significant differences. An average latency time of 9.55±0.90s was recorded for the PAPIN-KO with 9.84±0.71s for the PAPIN-control (P>0.05).

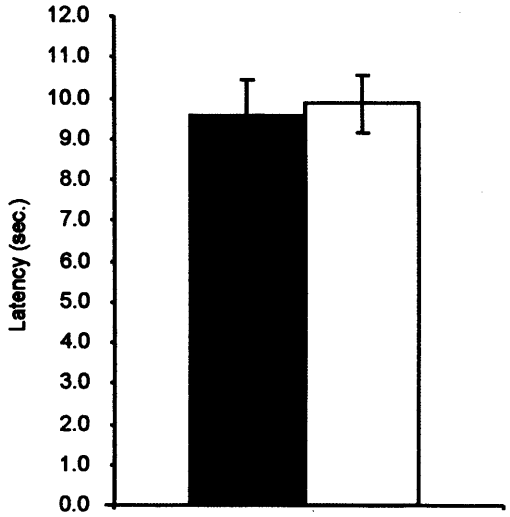

Figure 47. Acute pain responses to thermal heat.
■ PAPIN-KO, □ PAPIN-control

5.2.2.4 Formalin

Formalin was injected into the hind paw to record inflammatory responses (Fig. 48). Seven of each PAPIN-KO and PAPIN-control mice were tested showing no significant difference in responses respectively. The PAPIN-KO showed an overall second phase response of 142±42.42s compared with 133.30±28.64s for PAPIN-control mice (P>0.05).
Also, no significant difference was recorded during the first phase (P>0.05).

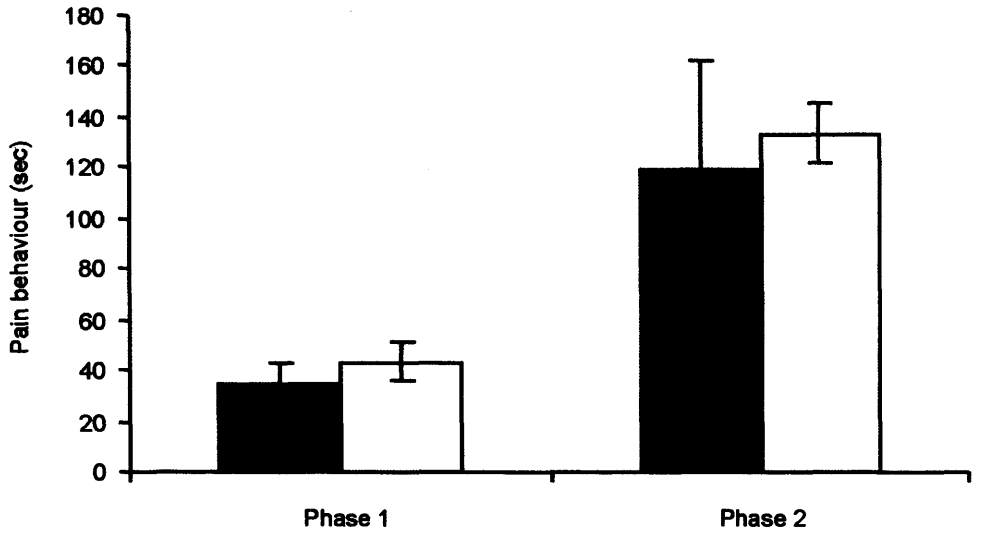

Figure 48. Responses to formalin
Responses divided into phase 1 (0-10 min and phase 2 10-60 min).
■ PAPIN-KO, □ PAPIN-control

5.2.3 Electrophysiology

The electrophysiology was investigated by Dr. Francois Rugiero.

Whole cell patch clamping was performed on DRG cultures prepared for both PAPIN-KO and PAPIN-control littermates; in total 61 large neurons and 15 small neurons were clamped.

To characterise the cells, electrical stimulation was used to distinguish between Low Threshold Mechanoreceptors (LTM) and High Threshold Mechanoreceptors (HTM) – see fig.48.

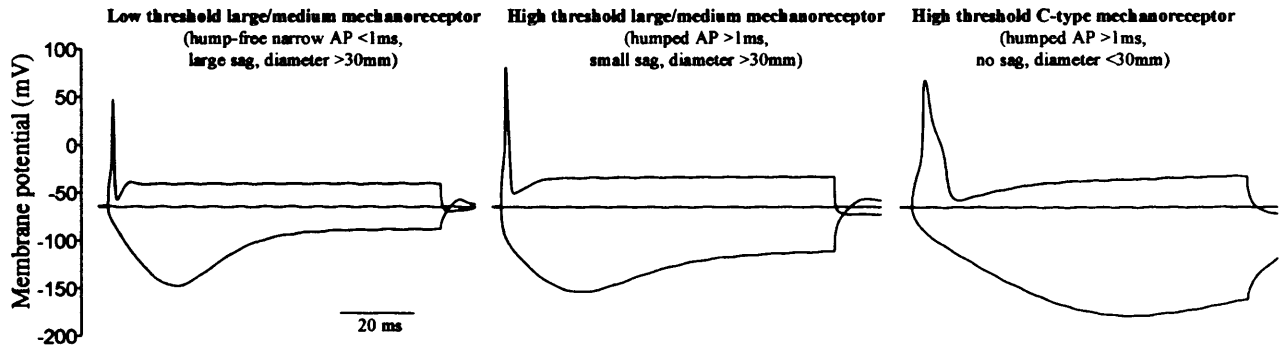

Figure 49. Membrane potentials for mechanoreceptors
The LTM is characterised by a narrow action potential, no shoulder, a small sag and fast repolarisation. One group of HTM generates a wider action potential, no shoulder, large sag and a slower repolarisation. Finally, the second group of HTM generates a very wide action potential, a shoulder, a very slow repolarisation and very large sag.

When analysing the data from the narrow AP, LTM, 21.5% of the PAPIN-KO cells were identified as non-responders compared with 0% from PAPIN-control. Further, the rapidly adapting cells were reduced from 85% in the PAPIN-control to 57% in the PAPIN-KO.

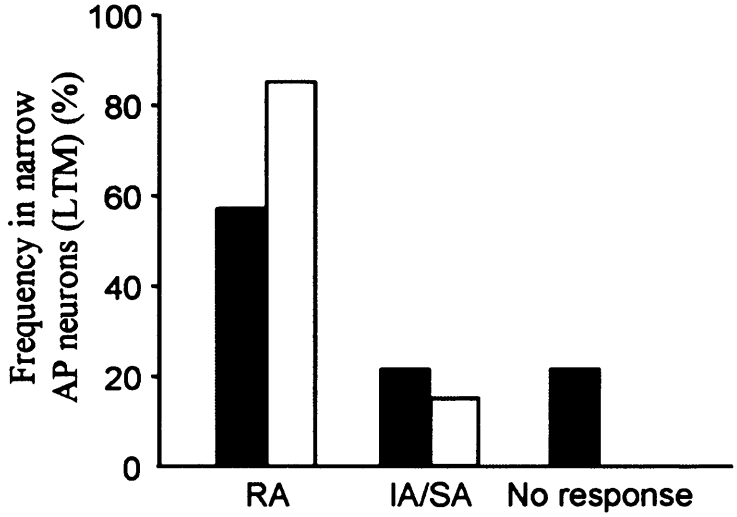

Figure 50. Characterisation of the LTM neurons.
Interestingly, 20% were non-responding in the PAPIN-KO.
■ PAPIN-KO □ PAPIN-control

When looking at the group HTM no differences were identified for the C-type. For the medium/large type, the number of non-responders was reduced from 69% in the PAPIN-control to 33% in the PAPIN-KO giving rise to a greater proportion of responding cells. This suggests that a phenotypic change or compensatory mechanisms are in place to adjust the balance as non-responding LTM cells were identified.

5.2.4 Comparison of cell expression

Sections were stained with anti-neurofilament and anti-peripherin to label large cells and small cells for comparison of cell expression between the PAPIN-KO and PAPIN-control. Interestingly, the PAPIN-KO (n=2) showed a lower expression of the neurofilament stained large neurons compared with the PAPIN-control mice (n=2). The average neurofilament positive cell count was 218±23 cells for the PAPIN-KO and 280±14 cells for the PAPIN-control ($P<0.05$). No difference was found for the peripherin positive cells, PAPIN-KO: 498±36 cells and PAPIN-control: 479±56 cells ($P>0.05$).

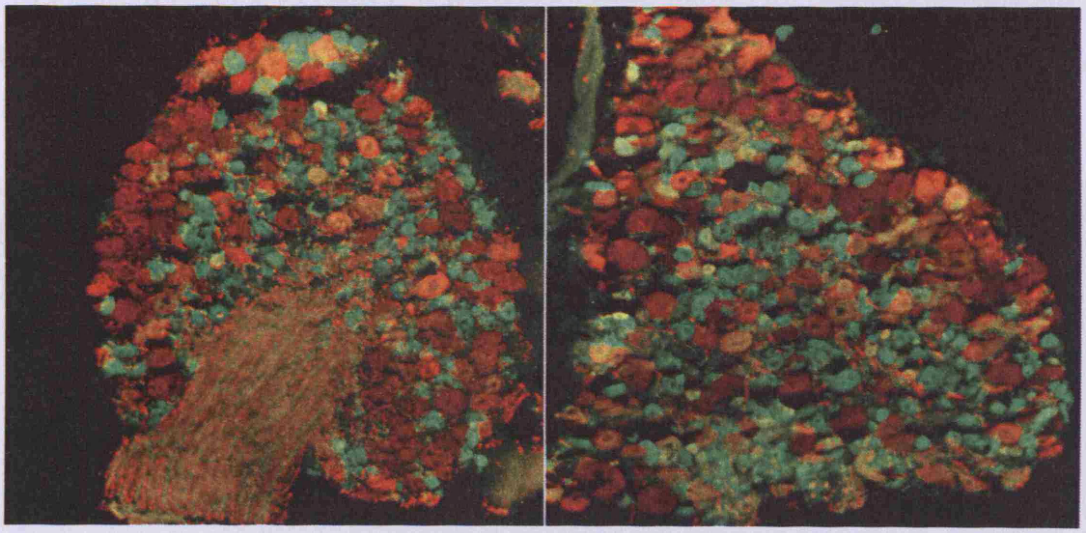

Figure 51. Papin staining for cell comparison
PAPIN-KO (left) and PAPIN-control (right),
Peripherin (green) and Neurofilament (red).

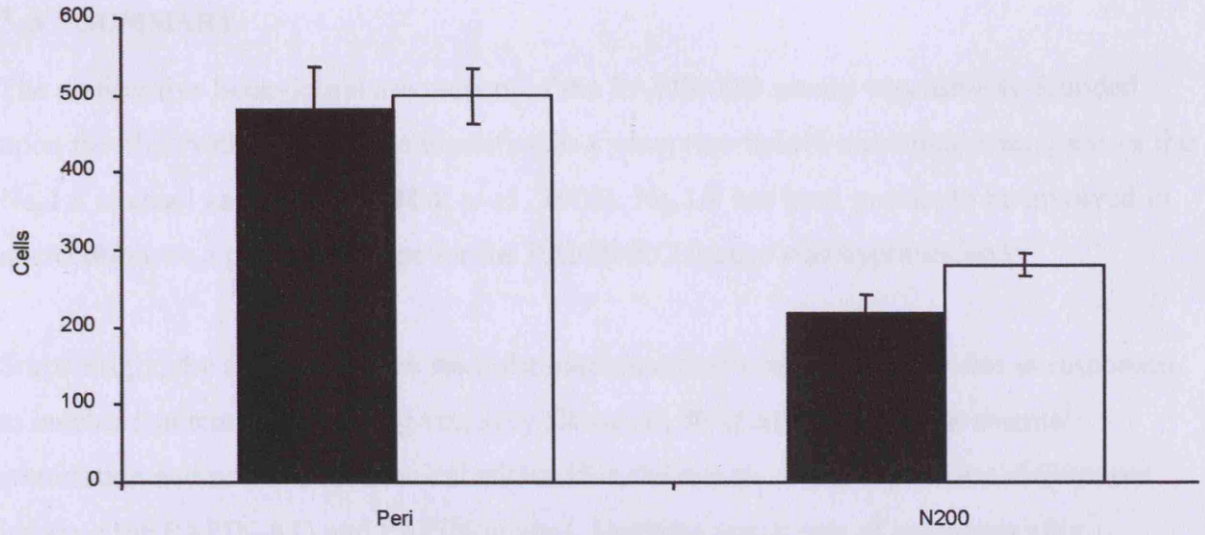

Figure 52. Cell comparison of the large and small neurons.
■ PAPIN-KO, □ PAPIN -control

5.2.5 Loss of neuronal connection in PAPIN-KO

Skin tissue from PAPIN-KO and PAPIN-control were stained for Merkel cells and Aβ-fibres (neurofilament).

The staining of skin was performed by Dr. Min Liu, Queen Mary University of London.

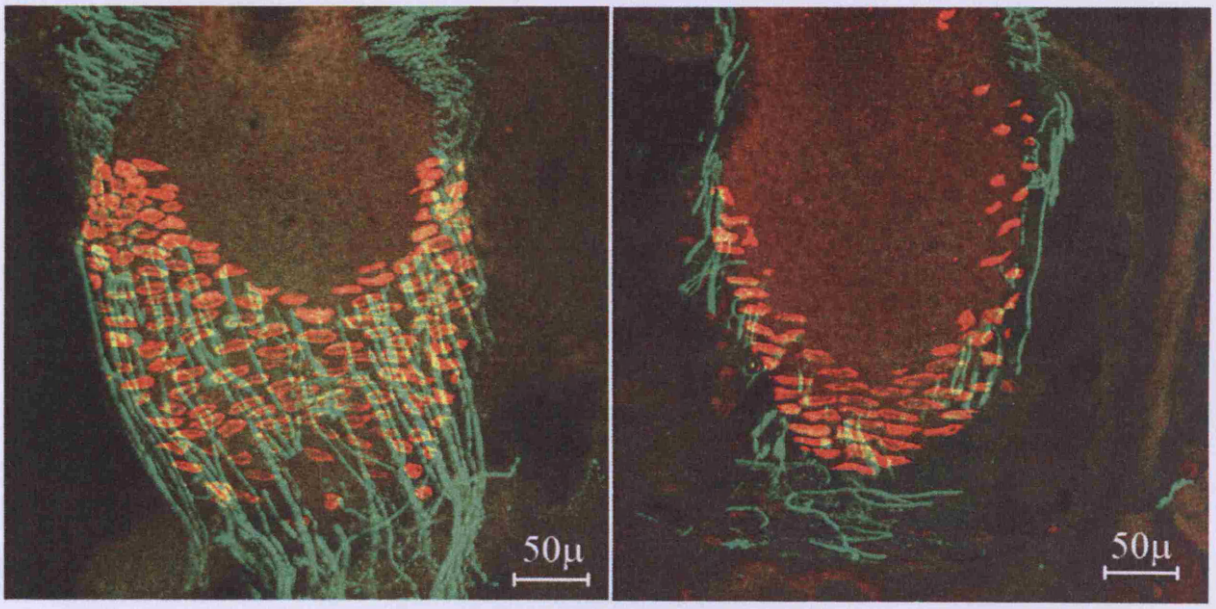

Figure 53. Lack of merkel cells and neuronal connection in PAPIN-KO
■ PAPIN-KO, □ PAPIN -control

5.3 SUMMARY

The nociceptive behavioural assessment of the PAPIN-KO mouse was initially founded upon the observation that it was identified in a yeast two-hybrid screening using parts of the $Na_v1.8$ channel as bait (Malik-Hall *et al.*, 2003). $Na_v1.8$ has been proven to be involved in nociception, so a pain phenotype for the PAPIN-KO mouse was hypothesised.

Surprisingly, the only difference from the assessment of acute thresholds was in responses to mechanical stimulation using von Frey filaments. Responses to noxious thermal stimulation and noxious mechanical stimulation did not show any significant differences between the PAPIN-KO and PAPIN-control. Further comparison of responses after induction of inflammation also did not show any differences.
This suggested an involvement of Papin in low threshold mechanotransduction rather than previously hypothesised, in nociception.

To investigate the involvement of Papin in low threshold mechanosensation, patch clamp electrophysiology was performed. Cells were divided on the basis of characteristics to electrical stimulation before a blunt glass probe was applied, stretching the cell membrane. Interestingly, of all the LTM cells which normally respond to mechanical stimulation 21% did not respond.
Furthermore, the testing of the medium/large HTM group a higher percentage than normal responded to mechanical stimulation, suggesting some form of compensatory activity.

An observation from the cultures of DRG was that the number of large neurons was reduced in the PAPIN-KO, leading to an immunohistochemistry comparison of neurofilament and peripherin positive cells, labelling the large/medium cells and small cells respectively. Interestingly, the analysis confirmed the observation from the DRG cultures. The number of neurofilament positive cells in the PAPIN-KO was reduced with around 20% compared with the PAPIN-control.

6 $NA_V1.7$ INVOLVEMENT IN PAIN

6.1 INTRODUCTION

The voltage gated sodium channel, $Na_v1.7$ is highly expressed in the small sensory neurons (Toledo-Aral *et al.*, 1997) and several lines of research have highlighted its importance as a possible drug target for the treatment of pain.

6.1.1 Congenital indifference to pain (CIPA)

Historically it has been reported that some humans are capable of withstanding immense stimuli of pain without displaying any sensation of pain. Congenital indifference to pain (CIPA) is a rare inheritable disorder, which is characterised by an absence of pain perception, but normal sensation to light touch (Swanson, 1963). It belongs to a group of rare clinical and genetic neuropathies of the peripheral sensory and autonomic nervous system. Along with insensitivity to noxious input, due to a severe loss of C-fibres, patients also suffer from moderate to severe mental retardation and anhidrosis (Shatzky et al., 2000). The disorder has been linked to mutations in the gene encoding the receptor for NGF, TrkA being responsible for the lack of survival of nociceptors (Indo et al., 1996).

More recently, a number of individuals were identified with a similar phenomenon of inability to perceive pain. Interestingly, compared with CIPA, under examination the subjects seemed to exhibit a completely normal physiological and mental development. The only physiological reason for their inability to feel pain is a distinct homozygous nonsense mutation in the gene encoding the $Na_v1.7$ eliminating expression of any functional $Na_v1.7$ channels (Cox *et al.*, 2006).

6.1.2 $Na_v1.7$ and Pain

The chronic inflammatory dominant human condition primary erythermalgia has been mapped to the $Na_v1.7$ gene and several different mutations described (Yang *et al.*, 2004;Waxman and Dib-Hajj, 2005;Cheng *et al.*, 2008). Another mutational study showed involvement in the inherited condition, Paroxysmal extreme pain disorder (PEPD, first

described as familial rectal pain). Different mutations in the sequence encoding the $Na_v1.7$ resulted in the painful condition characterised by paroxysms of rectal, ocular, or submandibular pain with flushing (Fertleman *et al.*, 2006). A third painful and disturbing condition, Urge Faecal Incontinence (UFI) has also been linked to $Na_v1.7$. Around half of patients suffering from UFI also suffer from rectal hypersensitivity. In a small study it has been reported that the expression of $Na_v1.7$ nerve fibres was increased in all layers of the rectal tissue (Yiangou et al., 2007). The reason for this is uncertain, but it has been suggested that an increase in NGF could be responsible as it regulates $Na_v1.7$ and $TrpV_1$ which is also reported upregulated in UFI (Yiangou *et al.*, 2007;Chan *et al.*, 2003).

6.1.3 The $Na_v1.7$ knockout mouse

In contrast to the above-mentioned human observations, a study generating a mouse $Na_v1.7$ knockout was found to die shortly after birth (Nassar *et al.*, 2004). When the knockout was directed to the DRG using the tissue-specific $Na_v1.8$Cre mouse, the behavioural assessment showed a strong involvement of the $Na_v1.7$ in acute and inflammatory pain (Nassar *et al.*, 2004). When tested for responses to neuropathic pain, no differences were found between the knockout and control (Nassar *et al.*, 2005).

6.1.4 Pharmacological blockers of $Na_v1.7$

One way to manipulate the function of proteins is using pharmacological blockers. This has, for many years, been a tool to investigate blockage of proteins as it is inexpensive and faster than generating transgenic mice. In the pharmaceutical sector, the synthesis of selective blockers has also been high on the agenda for development of potential drugs. Unfortunately, compounds have often turned out less specific than first expected. The recent discovery of the involvement of $Na_v1.7$ in nociception has not impeded the desire to generate a selective blocker for $Na_v1.7$. Several non-specific sodium blockers already exist, but a $Na_v1.7$ specific blocker could be potentially very beneficial.

6.1.5 Pharmacological agents

New structures have been synthesised and one of the new classes of blockers is the 1-benzapin-2-ones. They have been characterised and found to be both more potent and more

selective than compounds tested previously. More importantly, they have showed good bioavailability and efficiency when tested in neuropathic pain models on rats (Hoyt et al., 2007;Williams et al., 2007).

6.1.6 Venom derived agents

An interesting source of compounds with potential for drug use is toxins from spiders, scorpions, snails and snakes amongst others. The toxins consist of a mixture of up to several hundred different peptides, targeting many different receptors simultaneously, optimising methods of prey capture and protection. After isolating and purification and further characterisation using mass spectrometry some of the toxins have proven useful for treatment of several conditions, some types of pain and also as lead structures in the search for new drugs.

6.1.7 Conotoxins

Conotoxins are isolated from marine cone snails and have proven useful in the inhibition of voltage gated ion channels (Ekberg et al., 2007). The first approved "toxin" drug on the market was the Voltage Gated Calcium Channel (VGCC) N-type blocker, the ω-conotoxin MVIIA (Ziconotide, Prialt™) for the treatment of intractable pain (Lyseng-Williamson and Perry, 2006).

6.1.8 Spider toxins

In particular two different families of compounds are represented in the venoms from spiders: acylpolyamines and peptides. A host of both peptides and acylpolyamines have been isolated and described for blocking abilities for several ion channels (for review, (Estrada et al., 2007)).

Decade of studies into spider toxins have identified a number of venoms from tarantula spiders belonging to the *Theraphosidae* family (Escoubas and Rash, 2004). Several of the toxins characterised affect different ion channels. The *Psalamopoeus Cambridgei* toxin 1 (PcTx1) was isolated and characterised. It blocks the ASIC1a ion channel activating the endogenous enkephalin pathway. The results from pain models exhibit a remarkable

antinociceptive effect in both acute and inflammatory models as well as in the CCI model of neuropathic pain (Mazzuca et al., 2007). Several other toxins have been isolated and shown to modulate voltage gated sodium channels (Escoubas and Rash, 2004;Bosmans *et al.*, 2006).

6.1.8.1 The Tarantula toxin PhlTx1

More recently Phlotoxin 1 (PhlTx1) isolated from the genus *Phlogiellus* was reported as a potent blocker of the $Na_v1.7$ ion channel showing great selectivity for the $Na_v1.7$, IC_{50} value: 260nM. All other VGSC failed to respond at IC_{50}-values of up to 2.0μM. In the formalin test PhlTx1 produced a strong analgesic effect (Escoubas et al., 2006)

6.2 *IN VIVO* INVESTIGATIONS OF THE $NA_V1.7$ BLOCKER PHLTX1

Responses to acute thermal and mechanical stimulation were tested using Hargreave's instrument and von Frey filaments. Mechanical responses to long term inflammatory stimulation were tested by using CFA to induce an inflammatory response and test for hyperalgesia using von Frey filaments. Finally, investigations of mechanical responses to neuropathic pain were tested inducing neuropathic pain by partial ligation of the sciatic nerve (Seltzer) then testing for hyperalgesia using von Frey filaments.

6.3 RESULTS

C57/BL6 mice (WT) were used for testing the toxin. The toxin was diluted in saline to a final concentration of 1.0 nmole/10μL. As control saline was injected without any toxin. All experiments were performed blind to the injected substance.

6.3.1 Acute thermal sensation not affected by PhlTx1

12 WT mice were injected with either 1.0 nmole (10μL) of toxin or saline (10 μL) into the left plantar surface of the hind paw before testing.

No differences between the two injected groups were observed with latency in response for the saline group of 7.43±1.17s and 8.03±1.43s for the toxin group. ($P>0.05$).

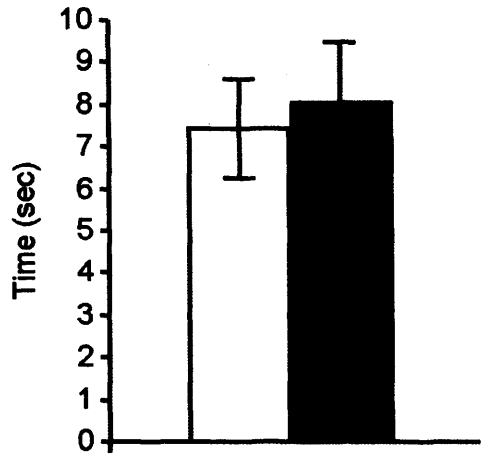

Figure 54. Responses to thermal stimulation.
■ Toxin, □ Saline

6.3.2 PhlTx1 significantly affects response to light mechanical stimuli

Responses to non-noxious mechanical stimulation were tested using von Frey filaments (Fig. 55). 12 WT mice were injected as described above before the von Frey test was performed.

When 1.0 nmole PhlTx1 was tested, a strong significant difference was monitored between the two groups. The saline group showed a 50% threshold of 1.68±0.39g compared with the WT group that showed a 50% threshold of 0.32±0.11g. (P=0.0114).

As a preliminary experiment, 0.2 nmole PhlTx1 was also tested on a small number of mice (n=4), only 1 mouse was injected with saline and 3 mice received the toxin. The result showed a 50% threshold for the toxin group of 0.88±0.12 g compared with 0.40 g for the control mouse. (For the graph, WT data from the original experiment was included).

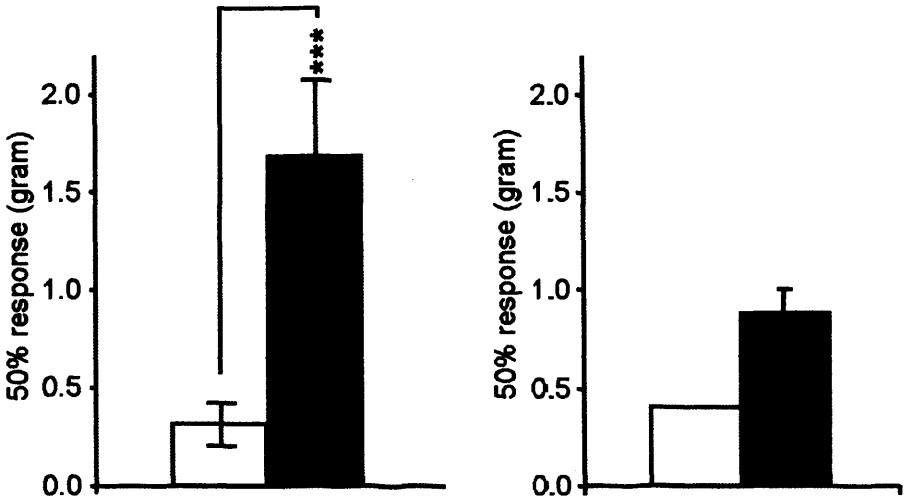

Figure 55. Responses to von Frey filaments
1.0 nmole PhlTx1 (left)
0.2 nmole PhlTx1 (right)
■ Toxin, □ Saline

6.3.3 Complete Freund's adjuvant

10 mice were used for the CFA test over a period of 4 days.

On day 0 baselines were recorded for responses to thermal (Hargreave's instrument) and mechanical (von Frey) stimulation, before injecting 20μL CFA into the plantar surface of the left hind paw.

On day 2 the mice were tested again and on day 3 the mice were injected with either toxin or saline as described above and tested again. Day 4, the mice were tested for a final time – see fig.54.

6.3.3.1 CFA – thermal thresholds

After treatment with CFA the mice tested on day 2 exhibited an increased sensitivity to heat compared with the baseline recordings, (baseline: 8.66±1.14s, Day 2: 4.86±0.58s). On day 3 the saline group still showed high sensitivity with a latency time of 5.15±0.49s compared with the toxin treated group that showed an average latency time of 8.26±0.81s. When tested again on day 4 both groups showed a high sensitivity (saline: 4.42±0.63s and toxin: 5.23±1.00s).

6.3.3.2 CFA - von Frey

After introduction of inflammation the mice showed a strong decrease in mechanical sensitivity when measured on day 2 (50% threshold, baseline: 0.8±0.05g, Day 2: 0.43±0.17g). Day 3, the saline mice were still more sensitive than baseline (0.66±0.19g) compared with the toxin group which showed a 50% threshold of 1.97±0.33g. When tested on day 4 both groups showed responses of higher sensitivity than compared with baseline values (saline: 0.44±0.08g, toxin: 0.27±0.07g).

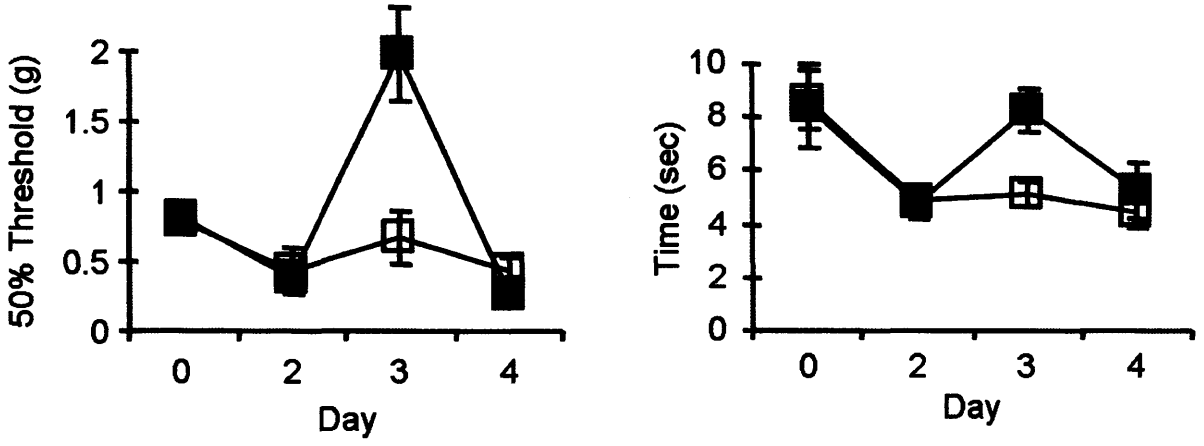

Figure 56. CFA - inflammation
Responses to von Frey filaments (left).
Responses to Hargreave's instrument (right).
■ Toxin, □ Saline

6.3.4 Neuropathic pain – Seltzer model

To test the effect of the PhlTx1 after neuropathic pain, 10 wild type mice underwent Partial Nerve Ligation (PNL). Thresholds to von Frey filaments were recorded before surgery and after surgery, the latter testing for mechanical hyperalgesia. At day 14 after surgery 9 mice were again tested before injections of either saline (5 μL) or toxin (0.5 nmole in 5μL) were injected. The mice were left for around 30 minutes before being retested.

Baseline data before surgery showed an average 50% threshold of 0.63±0.09g (n=10). On day 14 the 50% threshold was 0.15±0.02g (n=9) and after injections the response was 0.39±0.06g (n=4) for saline and 0.71±0.13g (n=5) for the toxin group.

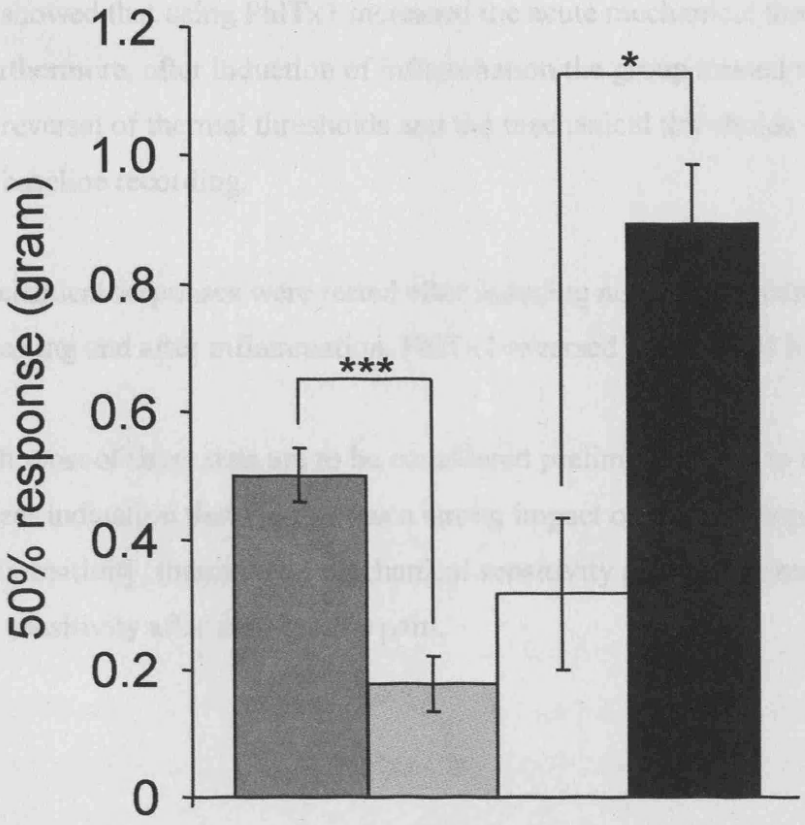

Figure 57. Mechanical responses after PNL.
■ von Frey baseline pre-surgery.
■ von Frey measurements after surgery.
□ von Frey measurements for saline-group.
■ von Frey measurements for toxin-group.

6.4 SUMMARY

After the discovery of the enormous impact a functional mutation of the $Na_v1.7$ has on the development and sensation of pain, the search for a selective blocker began. Historically, venoms from snails, spiders, snakes etc. have proven useful for the treatment of several conditions and a toxin from the Tarantula spider genus *Phlogiellus*, PhlTx1 was isolated and found selective for $Na_v1.7$.

The toxin was tested on mice for responses to mechanical and thermal stimulation, testing for both acute responses and to mechanical stimulation after introduction of inflammation.

The results showed that using PhlTx1 increased the acute mechanical thresholds, but not thermal. Furthermore, after induction of inflammation the group treated with PhlTx1 exhibited a reversal of thermal thresholds and the mechanical thresholds were reversed beyond the baseline recording.

Finally, mechanical responses were tested after inducing neuropathic pain. As seen in both acute pain testing and after inflammation, PhlTx1 reversed mechanical hyperalgesia.

Even though most of these data are to be considered preliminary, due to the low n-numbers, there is a clear indication that PhlTx1 has a strong impact on the development of acute mechanical sensitivity, thermal and mechanical sensitivity after inflammation and to mechanical sensitivity after neuropathic pain.

7 DISCUSSION

Three different ways to investigate mechanisms of nociception have been approached in this thesis. First of all, the deletion of the $Na_v1.8$-expressing neurons revealed that this population is essential for transmission of mechanical, cold and inflammatory pain, but not neuropathic pain or heat sensation. Secondly, the ubiquitously expressed protein Papin was demonstrated to be involved in touch. Finally, pharmacological blockage of $Na_v1.7$ proved efficient in preventing mechanical hyperalgesia after neuropathic pain.

7.1 ASSIGNMENT OF PAIN MODALITIES

Throughout the years hypotheses attributing particular pain modalities to specific plasma membrane cell receptors or ion channels have been studied as it is clear that the sensory neurons responsible for carrying the information about tissue damage (nociceptors) are polymodal (Perl, 2007). Some of the most extensively studied ion channels are the Trp-channels, $TrpV_1$ being the first cloned followed by other Trp-channels assigned to the sensation of heat. The attribution has been hypothesised partly on the background of electrophysiological studies, but when transgenic mice were generated a more complex picture emerged as in the case $TrpV_1$.

Another way to investigate and assign pain modalities to neuronal subtypes is by using some of the markers for specific subpopulations of sensory neurons such IB4 (non-peptidergic) and CGRP (peptidergic).
Over a decade ago, when the division of nociceptors was discovered due to their responsiveness to neurotrophic factors, studies suggested the NGF-dependent subpopulation (the peptidergic) was responsible for conveying inflammatory information as NGF was released following inflammation and the increased release of substance P and BDNF (Snider and McMahon, 1998). Furthermore the peptidergic neurons showed a significantly higher responsiveness to each of protons and capsaicin compared with IB4-positive neurons, but in contrast, after exposure to protons the response of IB4-positive neurons to capsaicin was more pronounced, and that of the peptidergic neurons less pronounced (Cunha *et al.*, 2005a). Later on, another study from Cheryl Stucky

demonstrated that this change in sensitivity is partly due to an upregulation of $TrpV_1$ in the IB4-positive neurons, when exposed to protons, and is partly the reason why IB4-positive neurons also contribute to inflammatory nociception (Breese et al., 2005). Together, these few examples demonstrate that it has not so far been proven that specific subpopulations of nociceptors can be assigned to specific pain modalities.

In this PhD thesis I have investigated the functional changes in nociception after ablation of the $Na_v1.8$-expressing neurons, a large population of the small sensory neurons and further identified a number of small sensory neuron specific genes that could be new targets in future treatment of several pain modalities.
To direct the ablation, the Cre/*loxP* system was used express diphtheria toxin tissue specifically in the $Na_v1.8$-expressing population (Ivanova *et al.*, 2005;Stirling *et al.*, 2005) as $Na_v1.8$ is expressed mainly in the small sensory neurons (Djouhri *et al.*, 2003).

7.1.1 Successful generation of the DTA-CRE and DTA-control mouse

Before any experiments were performed *in vivo* or in transgenic tissue, mice were assessed for any obvious differences in weight gain, motor function, fertility and general behaviour. No differences in weight gain and motor function were observed between the DTA-CRE and DTA-control; litters were all within expected sizes and the overall ratio of male/female progeny was close to 1:1. During the study a few mice had to be culled due to injuries. As recorded during the behavioural assessment, the DTA-CRE mice were insensitive to some pain modalities and tissue damage due to fighting or caressing could occur. To keep the experimenter blind to genotype and also to keep the experiments within ethical boundaries, these mice were culled.

The expression of Cre, mediating the expression of diphtheria toxin fragment A in the DTA-CRE mouse proved an efficient method of deletion of around 88% of nociceptors. Immunohistochemistry showed almost complete deletion of the $Na_v1.8$-expressing neurons. This accords with the results of previous experiments using the same floxed DTA-mouse, where cell ablation was shown to follow the pattern of Cre expression (Ivanova *et al.*, 2005).

One problem experienced previously from the use of diphtheria toxin is leakage of the toxin into the surrounding tissue. By using only fragment A of the diphtheria toxin no further cell death will occur when the targeted cells die and DTA leaks from the cytoplasm into the extracellular matrix. Diphtheria toxin is a two-fragment toxin and in the DTA mouse only fragment A is present. The absence of fragment B, the part responsible for internalisation upon binding to its receptor, prevents further inhibition of the protein synthesis in DTA-CRE mouse (Collier, 2001;Ivanova *et al.*, 2005).

7.1.2 No eGFP fluorescence detectable in the DTA-CRE or DTA-control mouse

From the immunohistological analysis, one important observation was the lack of fluorescence from the DTA-CRE and DTA-control mouse. The generation of the DTA mouse DNA construct included an enhanced Green Fluorescence Protein (eGFP) cassette that was inserted into the floxed part of the construct to detect and confirm the expression pattern. From any tissue expressing the modified gene (the floxed DTA construct, see Fig. 10, page 69), green fluorescence should theoretically be detectable after exposure to UV-light at wavelength 488nm. Any cells exposed to Cre would have lost the DNA fragment flanked by loxP sites including the eGFP cassette and the stop-signal preventing DTA expression. When any other tissue from the DTA-CRE or DTA-control mice was exposed to UV-light from the green spectrum, no visible fluorescence was detected.

The reason for lack of green fluorescence thus detected is probably due to the signal being too weak for detection as reported from other studies (Gusella *et al.*, 2002). Only by staining with an antibody against eGFP would it be possible to visualise the GFP. In this project it was not of any importance to see the eGFP fluorescence and I did not try in any way to check if the eGFP was detectable. In fact, if fluorescence had been detectable from the eGFP cassette it would have conflicted with the IHC analysis when antibodies against neuronal subtypes or proteins were used and in some cases detected using green fluorescent secondary antibodies.

7.1.3 Efficient deletion of $Na_v1.8$ positive neurons

Staining of DRG sections using an anti-$Na_v1.8$ antibody was used to show an almost complete loss of $Na_v1.8$ positive cells in the DTA-CRE mouse, confirming the complete expression of Cre in the $Na_v1.8$-expressing cells using the $Na_v1.8$Cre mouse.

After this crucial experiment, validating the experimental model, deletion of the total population of small sensory neurons was investigated. Markers for peripherin, labelling the small unmyelinated C-fibres (Goldstein et al., 1991), showed that around ~88% of the peripherin positive neurons had been ablated. It has previously been shown that $Na_v1.8$ is expressed within a similar percentage of the small sensory neuron population (Djouhri *et al.*, 2003). Staining with anti-neurofilament labelling the myelinated sensory neurons further revealed a more subtle deletion of a subset of the medium/large sensory neurons. It has been shown that $Na_v1.8$ is expressed in a small subset of medium sensory neurons (Akopian *et al.*, 1996).

In conclusion, the use of the floxed diphtheria toxin fragment A mouse in combination with the $Na_v1.8$Cre mouse has proven effective in generating progeny with the $Na_v1.8$-expressing neurons ablated.

7.1.4 Only peptidergic neurons left in DRG of the DTA-CRE mouse

Further IHC analysis of the DTA-CRE mouse, using markers for the peptidergic and non-peptidergic subpopulations of C-fibres, revealed that the large majority of the non-peptidergic neurons were deleted with a small group of peptidergic neurons remaining. The results show that the two sub-populations of small sensory neurons, divided according to their expression of neuropeptides, have different co-expression of $Na_v1.8$. All of the non-peptidergic neurons express $Na_v1.8$, but a small subset, ~ 12% of the peptidergic neurons, does not express $Na_v1.8$.

Of the remaining peptidergic neurons, substance P was showed to be expressed in very few cells in the DTA-CRE mouse compared with DTA-control.

7.1.5 Only peptidergic neurons remain at the central terminal in the DTA-CRE mouse

The central terminals of the primary sensory neurons have been described clearly n previous studies (Snider and McMahon, 1998). To investigate if any central changes could be attributed to deletion of the $Na_v1.8$-expressing neurons, sections of the spinal cord dorsal horn were characterised for expression of peptidergic and non-peptidergic neurons.

The results showed that the staining reported in lamina I and outer lamina II of the dorsal horn using anti-CGRP (Snider and McMahon, 1998) were strongly reduced in the DTA-CRE mouse compared with DTA-control spinal cord sections supporting IHC observations from DRG sections.

The lack of IB4-positive neurons in the spinal cord also supported the results from the DRG. No IB4-positive neurons, designated to the inner lamina II, were identified from sections of spinal cord from the DTA-CRE mouse.

7.2 $Na_v1.8$-EXPRESSING NEURONS CONVEY NOXIOUS MECHANICAL INFORMATION

Behavioural responses to noxious mechanical stimulation were considerably disrupted in the DTA-CRE mouse. Previously published works have suggested a number of transducers involved in noxious mechanical detection. In a separate study using the $Na_v1.8$Cre mouse, $Na_v1.7$ was deleted tissue specifically in the majority of the small sensory neurons and responses to noxious mechanical stimuli were greatly reduced (Nassar *et al.*, 2004). Also, when $Na_v1.8$ was deleted, a similar, but not as strong phenotype was identified (Akopian *et al.*, 1999) and finally a $Na_v1.7$/$Na_v1.8$ double knockout further confirmed this observation suggesting that both ion channels were involved in noxious mechanosensation (Nassar *et al.*, 2005;Nassar *et al.*, 2004)

The modification in the DTA-CRE introduced not only deletion of $Na_v1.7$ and $Na_v1.8$ in the $Na_v1.8$-expressing neurons, but the whole neuron was ablated and as hypothesised, a strong phenotype to noxious mechanical stimulation was recorded.

$TrpA_1$ has been reported by several groups to be involved in noxious mechanosensation and is exclusively expressed in the small sensory neurons (Kwan *et al.*, 2006;Petrus *et al.*, 2007). Recently, a subtype-specific $TrpA_1$ pharmacological blocker was developed and shown to reverse mechanical nociception after inflammation (Petrus et al., 2007), further indicating some involvement in mechanosensation.

The microarray analysis also highlighted a number of transcripts which, being selectively expressed, are possibly involved in mechanotransduction. $Na_v1.7$ was found to be only slightly reduced, but as this sodium channel is expressed across the DRG the actual reduction in the small DRG neurons may have been masked (Sangameswaran *et al.*, 1997). $Na_v1.7$ is important in mechanosensitive neurons, but is unlikely to be the transducer. Transcripts for $TrpA_1$ was strongly downregulated suggesting that $TrpA_1$ could play a role in the conveyance of information about mechanical tissue damage, possibly that of mechano-transducer.

Finally, electrophysiological data obtained from WDR neurons confirmed further a clear difference after noxious mechanical stimulation of the periphery between the DTA-CRE and DTA-control mice. Recordings from the DTA-CRE mouse showed that only a fraction of electrical impulses was conveyed after stimulation.

In conclusion, the result strongly suggests that $Na_v1.8$-expressing neurons are responsible for detecting noxious mechanical stimuli.

7.2.1 No phenotype for light mechanical sensation

Sensation from light stimuli has for long been an area of great interest. From a pain perspective, light mechanical stimuli can turn into a very painful and debilitating experience in particular after nerve injury where allodynia can develop.

The large Aβ-fibres, responding to non-noxious mechanical stimulation (Almeida et al., 2004) were not affected by the introduction of diphtheria toxin and when tested for responses to light touch the DTA-CRE mouse did not show any differences compared with

the DTA-control mouse. The result confirms that $Na_v1.8$-expressing neurons are not responsible for light mechanical sensation.

IHC showed a 13% reduction in the number of myelinated sensory neurons as $Na_v1.8$ is expressed in a small subset of medium sized neurons ((Djouhri and Lawson, 2004)), but this small reduction in the number of Aδ-fibres was not detected from the behavioural analysis for light touch responses. Furthermore, the WDR electrophysiology confirmed the behavioural result as both brush and application of non-noxious von Frey filaments did not give rise to any differences in central input. The WDR recordings using thicker von Frey filaments did show a strong phenotype for the DTA-CRE muse, but only for filaments over 1 g. When such thick filaments are applied a noxious mechanical and not a non-noxious stimulus is applied.

7.2.2 No involvement of $Na_v1.8$-expressing neurons in acute heat sensation

Responses to thermal stimulation were tested using two different models, the Hot plate and the Hargreaves' instrument. Several reports on possible transducers of noxious heat have been reported with many of them expressed either partly or exclusively in the small sensory neurons. By investigating the DTA-CRE mouse further information about subsets of sensory neurons involved in thermal sensation would emerge.

The response to the hot plate revealed minimal differences between the DTA-CRE and the DTA-control. The hot plate exposes the mouse to supra-threshold heat and the responses are considered to be supra spinal involving voluntary rather than involuntary behaviour (Jensen and Yaksh, 1984).

A more specific exposure to heat comes from the radiant heat delivered by the Hargreaves instrument and results in a reflex withdrawal (Hargreaves *et al.*, 1988). Interestingly, no significant difference between the DTA-CRE and DTA-control mice was recorded from the Hargreaves instrument. A trend in the responses to Hargreaves' instrument did suggest increasing resistance to heat in the DTA-CRE mouse, but the difference was not statistically significant. Previously, similar findings of a non-significant trend of increased

heat resistance were reported from the P11-KO, the $Na_v1.7$-KO and the $Na_v1.8$-KO (Foulkes *et al.*, 2006;Akopian *et al.*, 1999;Nassar *et al.*, 2004) suggesting that several proteins expressed in $Na_v1.8$-expressing neurons could influence thermal sensation. Surprisingly, from the $Na_v1.7/Na_v1.8$ double KO a pronounced resistance to heat was reported when using the Hargreaves instrument (Nassar *et al.*, 2005). This result was not reproduced testing the DTA-CRE mouse.

For long time several ion channels belonging to the Transient Receptor Protein family have been reported as the main transducers for noxious heat (Patapoutian *et al.*, 2003). $TrpV_1$ had been shown, using calcium imaging and electrophysiology, to be sensitive to capsaicin, heat and protons (Hek293 and Oocytes) (Caterina *et al.*, 1997). A few years later in 2000, two groups published *in vivo* data from $TrpV_1$ KO mice. One of the $TrpV_1$ KO showed similar results to the data previously obtained from electrophysiological recordings from transfected HEK293 cells and oocytes. Furthermore, the transgenic mouse showed a pronounced difference, compared to control, indicating a much suppressed response to heat in the hot-plate and immersion of the tail in hot water, but using the Hargreaves test no strong difference was found (Caterina *et al.*, 2000). After induction of inflammation using either CFA or mustard oil the subject again recorded a suppressed thermal response, but no phenotype was recorded when tested to mechanical stimuli (Caterina *et al.*, 2000). Also, no difference, thermal or mechanical, was observed after nerve injury (Caterina *et al.*, 2000). Data from another $TrpV_1$ KO ratified the inhibited response to heat and protons using electrophysiology (Davis *et al.*, 2000). Exposure to acute thermal stimulation using Hot Plate or Hargreaves' elicited indifferent response from the transgenic mouse compared with control. Only after introduction of inflammation, using carrageenan, was again a marked difference in response to heat noted (Davis *et al.*, 2000).

A more detailed analysis was performed by Woodbury et al. The study reported a strong difference in co-expression of $TrpV_1$ and IB4 (marker of the non-peptidergic neurons). In rat subjects, 50-55% of IB4-positive neurons also co-stained for $TrpV_1$. In contrast the number in mouse was found to be much smaller with only around 4% of the IB4-positive neurons co-staining for $TrpV_1$ (Woodbury *et al.*, 2004)

Using a skin-nerve preparation, heat sensitive fibres in the $TrpV_1$ KO mouse were identified and tested for responses to heat. With 57% of the WT and 78% of the KO fibres exhibiting a positive heat response, the data proved different to data published previously. The IB4-negative neurons were tested and overall there was no notable difference in heat responses when testing with and without $TrpV_1$ expression (Woodbury et al., 2004). To investigate further if $TrpV_2$ could be responsible, the heat responsive fibres from the $TrpV_1$ KO were stained with anti-$TrpV_2$. Only one out of twelve fibres tested positive for $TrpV_2$ further suggesting no involvement of $TrpV_2$ in the physical detection of noxious heat.

As $TrpV_1$ is strongly expressed in the $Na_v1.8$-expressing neurons my results do not support a role for $TrpV_1$ in acute noxious thermal sensation. Even though around 4.5% of the cells in the DTA-CRE mouse did respond to capsaicin compared with DTA-control using calcium imaging, it seems improbable that such a small number of positive cells would convey a normal response. The behavioural data from injections of capsaicin confirmed a lack of $TrpV_1$.

Data from the microarray experiment shows that $TrpV_1$ transcripts were downregulated 3.5 fold. The calcium imaging suggests the downregulation of $TrpV_1$ was greater. If the calcium imaging result is correct, a reason for the low expression of $TrpV_1$ in the DTA-CRE mouse could be caused by developmental changes after the ablation of the $Na_v1.8$-expressing neurons. Quantitative RT-PCR of $TrpV_1$ in the DTA-CRE was shown to be downregulated around 4-fold compared with DTA-control, similar to that measured in the microarray experiment.

In conclusion, the results indicate that $Na_v1.8$-expressing neurons may only contribute to sensation of noxious heat; arguably other non-$Na_v1.8$-expressing neurons are involved. One possible transducer reported to detect noxious heat is $TrpV_2$ (Dhaka *et al.*, 2006). The ion channel is expressed in medium and large neurons, is therefore not affected by the induced deletion by diphtheria toxin (Caterina *et al.*, 1999) and could contribute to heat sensation in the DTA-CRE mouse. In the microarray gene analysis $TrpV_2$ is neither down nor

upregulated, indicating that only minor regulation has occurred. No $TrpV_2$ KO transgenic data have been published to ratify *in vivo* involvement in thermal sensation.

7.2.3 Selective blockage of $Na_v1.7$ does not affect noxious thermal sensation

The tissue-specific deletion of $Na_v1.7$ in the $Na_v1.8$-expressing small sensory neurons gave rise to a trend towards thermal resistance, though non-significant when tested using Hargreaves' instrument (Nassar *et al.*, 2004). When a selective blocker of the $Na_v1.7$, PhlTx1, delivered locally to the paw, was tested using Hargreaves' instrument no differences in thermal sensation were recorded. Collectively, the data suggests that $Na_v1.7$ is not involved in the sensation of noxious heat.

7.3 Lack of sensation to noxious cold in DTA-CRE confirms a role for $Na_v1.8$ in cold responses

Recently, Zimmerman et al reported that $Na_v1.8$ is essential for the sensation of noxious cold (Zimmermann *et al.*, 2007). As $Na_v1.8$-expressing neurons have been deleted in the DTA-CRE mouse it could validate this observation. Interestingly, the DTA-CRE mouse did show a strong resistance in response to noxious cold using the cold plate. The data suggests that it is $Na_v1.8$-expressing neurons that convey noxious cold information.

When the DTA-CRE mouse was exposed to non-noxious coolness generated by the evaporation of acetone no difference was observed. $TrpM_8$ has been investigated for involvement in sensation of cold as it is located in the sensory neurons, expressed exclusively in a small subset of small neurons and activated by cold and menthol (Story *et al.*, 2003). When the $TrpM_8$ KO mouse was exposed to a range of temperatures, results showed enhanced resistance to cold below 15°C, suggesting that it does not detect innocuous cold, but noxious (Bautista *et al.*, 2007;Dhaka *et al.*, 2008)

In the microarray experiment, $TrpM_8$ was surprisingly not found to be up- or downregulated. To validate this result, qRT-PCR was performed and as with the microarray and functional behaviour, no difference was found versus control. A final investigation of

the $TrpM_8$ expression in the DTA-CRE mouse was performed using calcium imaging. Activation by menthol demonstrated that only a small proportion of the small sensory neurons in the DTA-control mouse are activated by menthol and this subset is almost intact even after deletion of the $Na_v1.8$-expressing neurons in the DTA-CRE mouse, suggesting that the $TrpM_8$ population is not co-localised with $Na_v1.8$.

Electrophysiological recordings from WDR neurons were performed and a range of temperatures applied to the paw. The temperature stimuli were applied using a syringe filled with water at the chosen temperature. This form of application elicits not only a thermal response, but also a mechanical response from the water-jet.

The results from the WDR electrophysiological analysis reproduced the behavioural observations showing no differences between DTA-CRE and DTA-control to any temperature applied. When 1°C water was applied in the WDR electrophysiology, no difference was found, but this is not comparable to the cold plate as the latter exposes the mouse to constant cold for 5 minutes. A more scientifically comparable behavioural assay is the acetone test, where evaporation of acetone applied for a short period cools the paw. On performing this test no difference was found between the DTA-CRE and DTA-control.

Contradicting the results from the DTA-CRE mouse in the above tests, results from WDR recordings from the $Na_v1.8$ null mouse did report a significant difference when responses to 1°C were measured (Matthews et al., 2006).

In conclusion, the results suggest that $Na_v1.8$-expressing neurons do not contribute to the acute thermal sensation, but are involved strongly after induction of inflammation. Also, $Na_v1.8$-expressing neurons are necessary for the detection of noxious cold.

7.4 $Na_v1.8$-EXPRESSING NEURONS ARE RESPONSIBLE FOR INFLAMMATORY PAIN

The neuronal response to tissue damage is a very complex situation. Inflammatory mediators sensitise the sensory neurons resulting in hyperalgesia and allodynia.

Some of the transducers expressed at the termini of the primary sensory neurons convey inflammatory pain. Deletion of $Na_v1.8$-expressing neurons was hypothesised to result in a reduction in responses to inflammation.
When the DTA-CRE mouse was injected with formalin, the measure of response was reduced significantly during the second phase, the phase believed to be inflammatory (Tjolsen *et al.*, 1992). When the DTA-CRE mouse was exposed further to long term inflammation, mechanical and thermal responses were measured for several days eliciting a strong resistance to both stimuli.

Of transducers involved in the sensation of inflammatory pain, $Na_v1.7$ is a strong candidate. In a previous study, $Na_v1.7$ was selectively deleted in the $Na_v1.8$-expressing neurons using the $Na_v1.8$Cre mouse. The result was a significantly reduced response in the second phase of the formalin test (Nassar *et al.*, 2004). This was confirmed further in responses to CFA, NGF and Carrageenan (Nassar *et al.*, 2004). Also, a delay in onset of inflammatory pain were measured in the $Na_v1.8$ KO (Akopian *et al.*, 1999). Finally, in the double knock out of both $Na_v1.7$ and $Na_v1.8$ the responses to inflammation were almost completely ablated (Nassar *et al.*, 2005). Both $Na_v1.8$ and $Na_v1.7$ expressed in the $Na_v1.8$-expressing neurons were deleted in the DTA-CRE mouse, ratifying previous observations.

$TrpV_1$ is another ion channel heavily involved in the development of inflammatory pain and is expressed in the sensory neurons (Davis *et al.*, 2000). The two sets of data published on $TrpV_1$ knockout mice show the response to heat after induction of inflammation were significantly reduced (Davis *et al.*, 2000;Caterina *et al.*, 2000).

$TrpV_2$ is also reportedly involved in the development of inflammatory pain. In wild type mice injected with either carrageenan or CFA, Probenocid, a selective activator for $TrpV_2$ induced a stronger licking and biting behaviour compared with wild type mice injected with placebo (saline) (Bang *et al.*, 2007). This suggests that activation of $TrpV_2$ affects the development of inflammatory pain. Interestingly, the $TrpV_2$ is mainly in the populations of medium and large sensory neurons and should not be affected by the diphtheria expression linked to $Na_v1.8$ expression. Therefore the data from the DTA-CRE mouse suggests that $TrpV_2$ is not involved in development of inflammatory pain. As previously mentioned, data from a $TrpV_2$ KO mouse has yet to be published.

One ion channel expressed in the small sensory neurons and reported involved in inflammatory pain is $TrpA_1$ (McNamara *et al.*, 2007). In the microarray gene analysis of the DTA-CRE mouse $TrpA_1$ is the most greatly downregulated Trp-channel (13.5 fold) so some involvement of $TrpA_1$ in inflammatory pain is very possible.

Among other ion channels of interest, $P2X_3$ has been showed to be upregulated after inflammation and to contribute to mechanical sensitisation (Dai et al., 2004). It was downregulated almost 12 fold in the DTA-CRE mouse, thus similar to $TrpA_1$ a role in inflammatory pain can not be excluded.

From the results, no specific receptor can be identified as responsible for the hyperalgesia observed after inflammatory pain, but for possible new drug treatment of inflammatory pain, receptors expressed in the $Na_v1.8$-expressing neurons should be targeted specifically as opposed to receptors such as $TrpV_2$ which are not expressed in $Na_v1.8$-expressing neurons. Finally, from the microarray list of regulated transcripts, a number of novel genes were found to be strongly downregulated in the DTA-CRE mouse, suggesting a possible role in the inflammatory pain seen in the DTA-control mouse.

7.5 $Na_v1.8$-EXPRESSING NEURONS ARE NOT RESPONSIBLE FOR NEUROPATHIC PAIN

Neuropathic pain mechanisms are not fully understood. The receptors, channels and sensory neuron populations that are involved in neuropathic pain pathogenesis have still not been characterised fully. Microarray experiments have been used to investigate changes in gene expression and have suggested the involvement of several hundreds of genes, in the DRG, spinal cord and surrounding tissue (Griffin *et al.*, 2007;Costigan *et al.*, 2002).

In the DTA-CRE mouse, no differences in mechanical or thermal thresholds were measured after introduction of nerve injury compared with DTA-control. In previous studies using the $Na_v1.8$Cre mouse to delete receptors in a tissue-specific manner similar results were obtained. $Na_v1.8$-expressing tissue-specific knockouts of $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ (each individual, and the latter two together) as well as P11 and BDNF (Zhao *et al.*, 2006;Foulkes

et al., 2006;Nassar *et al.*, 2004;Nassar *et al.*, 2006;Nassar *et al.*, 2005) demonstrated no involvement in neuropathic pain thresholds.

The data from the DTA-CRE mouse, together with previous data, suggests that sensory neurons other than $Na_v1.8$-expressing neurons are arguably responsible for the development and maintenance of neuropathic pain.

When transgenic mice have been tested for development of neuropathic pain, the gene modification has often been performed in a non-specific way. The $Na_v1.8$Cre mouse only deletes the target gene in neurons expressing $Na_v1.8$, leaving most medium and large primary neurons unchanged. This difference suggests further that fibres other than those expressing $Na_v1.8$ are required for the development of neuropathic pain.

Recently spinal cord slices were investigated using Calcium imaging to establish whether Aβ-fibre information could spread from lamina III to more superficial laminae after neuropathic pain (SNI) (Schoffnegger et al., 2008). The results showed that activation of only Aβ-fibres gave Ca^{2+} transients in superficial nociceptor specific laminae in 67% of the neuropathic rats compared with 12% for the control rats. Also, chemical and electrical stimulation of the deep dorsal horn resulted in activation of superficial laminae. Together with the previous observations and the *in vivo* data from the DTA-CRE mouse it seems very likely that Aβ-fibres play a very important role in the development and maintenance of debilitating allodynia and hyperalgesia seen with neuropathic pain.

7.6 $Na_V1.7$ SPECIFIC BLOCKER REVERSES NEUROPATHIC PAIN

The $Na_v1.7$-specific blocker, PhlTx1, delivered locally to the paw, was demonstrated to reverse the development of neuropathic pain. As reported, the $Na_v1.7$ KO, limited to the $Na_v1.8$-expressing neurons, did show a strong development of hyperalgesia after nerve injury suggesting that blockage of $Na_v1.7$ in neurons other than those expressing $Na_v1.8$ are responsible for neuropathic pain.

7.6.1 $Na_v1.8$-expressing neuron specific transcripts

Total RNA from DRG dissected from DTA-control and DTA-CRE mice was used for a microarray gene analysis to distinguish which transcripts had been either up- or downregulated. As the $Na_v1.8$-expressing neurons had been ablated, the microarray experiment would make it possible to identify transcripts enriched or specifically expressed in the $Na_v1.8$ positive neurons. Transcripts strongly enriched in the DTA-KO could mean an involvement in some of the remaining pain modalities and likewise strongly downregulated transcripts are possibly responsible for the suppressed modalities.

Whilst around half the probes on the gene chip are used for controls, the regulation of several transcripts also acted as controls as they are co-localised with $Na_v1.8$ e.g. $Na_v1.9$ (downregulated 23-fold) (Fang et al., 2002). $Na_v1.8$ itself should obviously be strongly downregulated and fortunately, this was the case (downregulated 37-fold).

Using microarray in this way will not identify all regulated genes. If the transcripts are expressed widely across the DRG some down-regulation can be expected. If the expression of a transcript is limited to only the small DRG a large down-regulation would be expected. Finally, an up-regulation would be expected if the transcript was expressed only in the large neurons. Further, for the microarray platform I used any splice variants of transcripts will not be detected. Also, the proportion of mRNA is not identical to the proportion of protein translated.

Sodium channels other than $Na_v1.8$ and $Na_v1.9$ are also regulated according to the microarray analysis. $Na_v1.7$ is only downregulated 2.2 fold, but this could be due to its expression in the large sensory neurons (Sangameswaran *et al.*, 1997). The widely distributed $Na_v1.1$ (upregulated 1.8-fold) has not been linked to nociception.
Two different β-subunits are regulated. The Na_v-type II β-subunit (downregulated 3.7-fold), modulates channel gating, assembly and expression and is expressed in all subtypes of DRG neurons (Chahine *et al.*, 2005). Na_v-type III β-subunit (downregulated 2.9-fold) is expressed in the small and medium DRG neurons (Chahine *et al.*, 2005), but has not been linked to nociception.

Several ion channels belonging to the Trp-family showed a reduction in transcript levels. $TrpA_1$, $TrpV_1$ and $TrpV_2$ and their involvement in nociception have been described previously. $TrpC_3$ and $TrpC_6$ (downregulated >6 fold) are each expressed in the small sensory neurons, but neither has been shown to be involved in nociception.

Of the calcium channels (and subunits) the transcript most downregulated in the microarray analysis is the $\alpha_2\delta$-1 subunit (downregulated 3.4-fold), predominantly expressed in the small sensory neurons (Yusaf et al., 2001) and is involved in neuropathic pain rather than acute pain (Li *et al.*, 2004). The calcium channel α-subunit, $Ca_v1.2$ (L-type) (downregulated 3-fold) has not been linked to pain. Of calcium channels involved in nociception, mostly belonging to the N-type (Ca_v2) or T-type (Ca_v3) families of VGCC (Gribkoff, 2006), only $Ca_v2.2$ was downregulated (3.2-fold) in the microarray experiment. Of the VGCC subunits the β3-subunit was downregulated 1.5 fold. From the study of the β3-subunit knock-out mouse it was shown that β3 was strongly involved in inflammatory pain, but only moderately involved in acute nociception (Murakami et al., 2002).

The G-Protein coupled receptors MrgA2 and MrgA3, both expressed in nociceptors (Dong et al., 2001) and possibly involved in modulating sensory information (Liu et al., 2008), were both downregulated (11-fold and 18-fold respectively) in the DTA-CRE mouse.

Transcripts for prostaglandin receptors, involved in inflammation (Matsuoka and Narumiya, 2007), were downregulated between 2- and 4-fold. $P2X_3$ (downregulated 11.9-fold) is involved in inflammatory responses due to the release of ATP and possibly in thermal sensation (Souslova *et al.*, 2000). As $P2X_3$ is exclusively expressed in small neurons a strong downregulation was expected in the DTA-CRE mouse (North, 2004). This would support the observation from the immunohistochemical characterisation showing that the non-peptidergic neurons had been ablated, as the $P2X_3$ channel is exclusively expressed in IB4-positive neurons (Burgard et al., 1999).

The serotonin receptor, $5\text{-}HT_4$ (downregulated 16-fold) is expressed throughout the sensory neurons, with its highest content in the small C-fibres (Nicholson et al., 2003), but without any direct links to nociception. Interestingly, $5\text{-}HT_3$ which is distributed in a similar way as $5\text{-}HT_4$ and involved in several pain modalities was found to be downregulated 2.1-fold in

the experiment. The 5-HT_{1A} receptor (downregulated 2.9-fold) has no reported influence on peripheral nociception, but is involved in several psychiatric disorders (Sharp et al., 2007). CGRP-R2 (downregulated 3.6-fold), one of the receptors for the neuropeptide CGRP, released upon a host of stimuli in the periphery and activates and sensitise nociceptors, is expressed in both central and peripheral neuronal tissue (Benemei et al., 2007).

Several of the markers for neuron subtypes are regulated in the DTA-CRE mouse. RET, the tyrosine kinase receptor for GDNF (Molliver *et al.*, 1997) is expressed in the IB4-positive non-peptiodergic neurons, but also in some large neurons (Bennett et al., 2000). RET is only downregulated 1.5-fold, but this could be partly due to the fact that it still is expressed in some large neurons and also possible compensation from an upregulation in the large cells. Another marker is the transcription factor RunX1, shown to be involved in the transition of a subpopulation of sensory neurons changing from expressing TrkA to RET with a distribution within the DRG appearing to be restricted to nociceptors (Molliver *et al.*, 1997;Theriault *et al.*, 2004). In the DTA-CRE mouse RunX1 is downregulated 4-fold. The other transcription factor regulated, belonging to the Runx-family, is RunX3 (upregulated 2.4-fold). RunX3 is involved in the differentiation of the large neurons so an upregulation is expected as the large neurons represents a larger proportion of the RunX3 in the DTA-CRE compared with DTA-control (Inoue *et al.*, 2002).

Of transcripts coding for receptors involved in endogenous analgesia, the μ-opioid receptor (downregulated 7-fold) is strongly linked to the $Na_v1.8$-expressing neurons. A study of a rare polymorphism of the μ-opioid receptors suggests involvement of the receptor in noxious mechanosensation (Fillingim et al., 2005).

Of particular interest, a group of transcripts, GPCRs, Kinases, channels and receptors has been identified that has not previously been associated with nociceptors, but associated with the $Na_v1.8$-expressing neurons. Among these transcripts must be receptors partly responsible for pain modalities absent in the DTA-CRE mouse – cold pain, noxious mechanotransduction and inflammatory pain.

One of the most regulated transcripts in the DTA-CRE mouse is the GPCR, GNA14 (downregulated 37-fold) belonging to the Gq subfamily of the GPCR superfamily and has

very recently been cloned from porchine (Chen et al., 2008). The Kelch-like 5 transcript (Klhl5 downregulated 17-fold) belongs to the Kelch protein super-family. Klhl1 is involved in trafficking and modulation of the VGCC currents $Ca_v2.1$ (Aromolaran et al., 2007) and Khlh5 is likely to have similar cytoskeletal characteristics.
Edg7 is another transcript showing an almost complete co-expression with $Na_v1.8$ (downregulated 44-fold) (Takuwa et al., 2002), but no published links to nociception and as it is expressed ubiquitously it is not a potential drug target.

The data obtained from the microarray experiment is a suitable basis for further experiments. The $Na_v1.8$-expressing neurons were deleted reducing the peripherin-positive cells by around 88 percent. This suggests that e.g. the transducer for one of the remaining pain modalities, heat must be either expressed within the remaining subset of non-$Na_v1.8$-expressing neurons or in another population of sensory neurons.

7.7 EXTRACELLULAR ELECTROPHYSIOLOGY CONFIRMS OBSERVATIONS

Investigations of the information carried through to the dorsal horn could possibly validate some of the observations obtained from behavioural studies. However, it is important to be aware of a few differences between the techniques. The electrophysiological experiments are performed *in vivo*, but the mice are under deep anaesthesia enabling the measure of supra-threshold stimuli of the neurons compared with the behaviour where pain thresholds are measured.
Also, the range of stimuli differs according to the measurement techniques performed for a given modality. For light mechanical stimulation using non-noxious pressure, a brush is used for the electrophysiology, whereas von Frey filaments are used for behaviour. For noxious mechanical pressure, pressure applied using forceps is used for electrophysiology but the Randall Selitto apparatus for the behaviour.

Thermal stimulation was tested at several different temperatures, cold, warm and hot. For the electrophysiological analysis all temperatures were applied using water compared with behavioural where hot-plate, Hargreaves', cold plate and acetone were used. These variants could give rise to different responses.

No electrophysiological recordings were performed after introduction of inflammation or nerve injury.

7.8 PAPIN

From the discovery that the Papin protein interacts with $Na_v1.8$ it was believed that it could have an effect on pain thresholds as seen with another $Na_v1.8$-interacting protein, P11 (Foulkes *et al.*, 2006). A Papin knock out mouse (PAPIN-KO) was investigated for acute pain thresholds and interestingly no differences were recorded when exposed to painful mechanical, thermal or inflammatory stimuli. In contrast, when tested for responses to non-noxious mechanical stimulation using von Frey filaments (Hogan, 2002) the 50%-threshold of the PAPIN-KO mouse compared with Papin control littermates (PAPIN-control) was more than doubled: 1.55g vs. 0.60g. This result was unexpected and not considered to be related to the interaction with $Na_v1.8$.

One way to investigate this observation further was by recording electrophysiological changes after mechanical stimulation of cultured DRG neurons. The low-threshold mechanoreceptors (LTM), identified by their narrow action potential, all respond to mechanical stimuli whereas only around 30% of the LTM characterised by wider action potentials are activated by mechanical stimulation (Drew *et al.*, 2004).
The results showed that in the PAPIN-KO around 20% of the LTM cells with narrow action potential did not respond. There could be several reasons. One is that the cells are mechanosensitive, but when the stretch of the cell membrane exceeds 7-8μm, the cell tends to die and a change in current is not identified. Another possibility is that the cells are in fact non-responders.

The other electrophysiologically interesting observation is the increment in the percentage of mechanically activated wide action potential LTM cells. Normally, around 70% of these cells will not respond to mechanical stretching of the membrane (Drew *et al.*, 2004), but in the PAPIN-KO mouse only 35% cells did not respond suggesting a significant change mechanically activation. This change can be due to a shift in phenotype of the cells to compensate for the difference seen with the narrow action potential LTM cells.

An observation made while performing the electrophysiology gave rise to the suggestion that the number of large neurons stained by anti-neurofilament, were lower in the PAPIN-KO compared with the PAPIN-control. Whole DRG were sectioned and stained to compare both the large cells and the small cells (anti-peripherin positive).
No difference in the number of small peripherin stained cells was found, but interestingly the number of large cells stained with anti-neurofilament showed a significant difference with 22% less cells expressed in the PAPIN-KO when compared with the PAPIN-control.

This result gives another possible explanation to the observed behavioural phenotype, as the numbers of conducting cells are reduced in the PAPIN-KO.

Finally, immunohistochemistry of the skin demonstrated significant differences between the PAPIN-KO and PAPIN-control in the anatomy of the cells. Strikingly, the physical connection between the merkel cells and the sensory neurons was lost in the PAPIN-KO mouse suggesting another possible reason for the lack of response to light mechanical stimulation.

In conclusion, a behavioural observation found that the PAPIN-KO was deficient in response to non-noxious mechanical stimulation. This observation was investigated by electrophysiology and immunohistochemistry and a number of possible explanations were identified.

- A greater number of non-responding narrow action potential LTM fibres were found in the PAPIN-KO compared with PAPIN-control.
- More responding wide action potential medium/large fibres were found in the PAPIN-KO
- A lower number of medium/large sensory neurons were found in the PAPIN-KO compared with PAPIN-control.
- A strong reduction in the connection between merkel cells and sensory neurons was identified in skin from PAPIN-KO.

7.9 Na_V 1.7 BLOCKAGE USING PHLTX1

The discovery of the importance of $Na_v1.7$ in nociception has been a breakthrough in the search for new treatments of pain hence research into this specific ion channel has increased significantly. In mouse, a DRG tissue-specific deletion of $Na_v1.7$ demonstrated a significant role of the sodium channel in mechanical and inflammatory pain. In human, a functional deletion of $Na_v1.7$ demonstrated a remarkable ablation of the ability to sense tissue damage; whereas mutations of $Na_v1.7$ have been linked to various painful conditions.

A toxin, PlhTx1 isolated from the Tarantula Spider was found to block $Na_v1.7$ selectively. Its ability to reduce responses a range of stimuli, acute, after introduction of inflammation and also after nerve injury, was tested *in vivo*.

No difference in response was found between the toxin group and control group when the injected paw was exposed to radiant heat. Only after inducing inflammation did the PhlTx1 reverse the thermal hyperalgesia recorded in the saline-treated control group. Responses after mechanical stimulation were greatly reduced by the toxin. The acute threshold to von Frey filaments was increased after injection of PhlTx1 and the post-inflammatory hyperalgesia was more than reversed.

Interestingly, mechanical hyperalgesia seen after nerve injury was also reversed in the group of mice treated with the toxin.

The data suggest that blockage of $Na_v1.7$ expressed in the sensory neurons can reverse several different pain modalities. Of particular interest is the observation after nerve injury where mechanical hyperalgesia was reversed as this debilitating condition has been proven difficult to treat with current drugs.

As the data collected is limited and the n-numbers small the data has to be considered carefully before drawing conclusions. To further investigate the role of $Na_v1.7$, one possibility would be to generate a transgenic mouse with deletion of $Na_v1.7$ not only in the small sensory neurons, but also in the medium and large sensory neurons. From this experiment mechanical allodynia after neuropathic pain should be prevented as seen with

PhlTx1. One transgenic mouse line expressing Cre in all the sensory neurons is the Wnt1-cre (Danielian *et al.*, 1998). Unfortunately Cre is also expressed in other neuronal tissue, but it would still be a way to investigate further the deletion of $Na_v1.7$ in primary sensory neurons.

8 CONCLUSION AND PERSPECTIVES

The transgenic approach to delete a specific subpopulation of sensory neurons has proven very successful in the investigations of pain modalities attributable to the $Na_v1.8$ subpopulation. The study combined behavioural analysis, extracellular electrophysiology and microarray gene analysis to investigate the transgenic mouse. The results showed that noxious mechanical hyperlagesia, inflammatory pain, but not thermal hyperalgesia or neuropathic pain are conveyed by the $Na_v1.8$-expressing neurons.

To investigate the data obtained further, potential candidates from the microarray data could be deleted using siRNA in DRG cultures and investigated using electrophysiology before generating a transgenic mouse line. Also, the skin-nerve preparation where the electrophysiological signalling from the periphery to the dorsal horn can be investigated could be an interesting approach to obtain more detailed information of e.g. which neurons in the DTA-CRE mouse is responsible for the noxious thermal sensation.

To investigate the DTA mouse line further, it could be interesting to examine the termination of Aβ-fibres at the dorsal horn. Aβ-fibres can be stained by injecting cholera toxin-B into the sciatic nerve. The toxin will bind to the myelinated fibres and after a couple of days of retrograde transport, the dorsal horn can be excised, stained and the termination will be visualised (Bennett et al., 1996).

8.1 THE USE OF TRANSGENIC MICE

In this Ph.D. project two different methods of interrupting gene expression and one of interrupting function were used. For the deletion of a subset of sensory neurons the Cre/*loxP* system was used. For the investigation of Papin a conventional technique was used generated by random LacZ insertion (gene trap) and finally pharmacological block of the $Na_v1.7$ ion channel using a spider toxin. Interestingly, these different approaches have each proven useful in identifying proteins involved in nociception and pain.

9 APPENDIX

Publications

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